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CANCER RESEARCH

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pH Studies of Malignant Tissues in Human Beings

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INTRODUCTION

On the basis of extensive studies, it has long been contended by many investigators that the hydrogen ion concentration of malignant tissues differs from that of normal tissues in a characteristic way. The results obtained, however, are highly controversial. Many workers have found an alteration of pH to the alkaline side (1, 4, 5, 9, 14, 16). Others have found an alteration to the acid side (7, 8, 13, 15, 20). Still others (2, 11, 23) have found no specific pH change in neoplastic tissues. These wide differences in results are explained by Stern and Willheim in several ways:

1. The hydrogen ion concentration of living tissue undergoes constant variations as a result of continuous metabolic processes. 2. Comparisons with values of normal tissues are few, in experimental animal work as well as with human tumors. 3. The technic used, the changes introduced by the method itself and the individual interpretation of results all contribute to the varying conclusions drawn as to pH alteration in neoplastic tissues.

Some workers have tested freshly prepared extracts of tumors by a colorimetric method while others have injected indicator substances into tumor-bearing animals. Both quinhydrone and glass electrodes have been used in tissue cultures, in material surgically-removed *in vitro* and in living, lightly anaesthetized animals *in vivo*.

Many, including Voegtlin, Kahler and Fitch (22) agree that the glass electrode is best suited for the purpose of study of this problem. It has long been demonstrated that the presence of oxidizing and reducing substances derived from tissues did not introduce any error in the pH determinations made by the bulb type of glass electrode (22). Voegtlin, DeEds, Kahler and Rosenthal (19) in 1929 demonstrated that the insertion of bulb type of electrodes into skeletal muscle would give rough estimations of pH changes under varying physiological conditions. Snell and Biffen (17), in discussing the advantages of the glass electrode, state that "the glass electrode is not affected by oxidation-reduction potentials in the solution. It has no salt or protein error and usually comes to equilibrium immediately. Most instruments have incorporated temperature compensators. Many types of glass electrodes are available and may frequently be used interchangeably with those in the original apparatus."

Our study was carried out at the Cook County Hospital in Chicago where pH determinations were made by means of the Beckman pH meter, Model G, using both glass bulb type and capillary-type electrodes (No. 1190 and No. 1190 × 5) on tissues freshly removed at surgery (Figs. 1 and 2). The Beckman 1190 × 5 glass electrode is built with a special pH sensitive glass bulb blown on the end of standard lead glass tubing. The bulb is subsequently shaped into a taper for ease of inserting into soft materials. The buffer solution used in the tip of the glass electrode is such that when this electrode is employed in combination with the standard 1170 Reference Calomel Electrode it provides the proper emf-pH characteristics to the Model G circuit so that the resulting measurements made with the instrument read directly in pH units. The solution used in the glass electrode does not deteriorate with age. In the pH range from 0 to approximately 9.5-10.0 pH, glass electrodes act quite accurately as a hydrogen electrode. The calomel electrode consists of a permanently filled, calomel-mercury internal, and an external body around it which is kept filled with saturated KCL solution. The liquid junction potentials between the solution under measurement and the KCL solution is achieved by adjustment of the concentrations of the components of the special filling solution used in the glass electrode. Because the composition of all of the solutions used in the two electrodes is always the same and the

glass used in the glass bulb is the same for each electrode, the EMF temperature relationship for all electrodes is the same. Therefore, a temperature-compensating electrical circuit is constructed within the instrument. This temperature compensator does not correct pH readings to a base temperature value but merely adjusts the circuit to read the proper pH at the temperature of measurement. For example, a pH measurement taken at 40° C. with the temperature compensator at 40° C. gives the actual pH of the solution at 40° C.

Although it was our original intention to test all specimens at a given time interval following surgical removal, local conditions in the operating rooms prevented this to a certain extent. The pH of neoplastic and other tissues was determined at periods ranging from 5 to 90 minutes after surgical removal. In all cases, multiple determinations were made on each sample and at several sites of the tissue. Many specimens were examined at repeated intervals in an attempt to evaluate the influence of time following removal on the pH of the specific tissue. Other factors considered and noted were the type of preoperative treatment (medication, x-ray and radium treatment of the tumor), type of anaesthetic and the administration of glucose, saline, plasma and blood during surgery. The Beckman apparatus was kept in a stationary place in a room on the surgical floor and all specimens were carried to the apparatus. The electrodes were standardized against phosphate buffer (specifically prepared for use with Beckman pH meter) at the specific room temperature before and after examination of each surgical specimen. The outer surface of the tissue to be examined was carefully dried, using tissue paper with neutral pH and a small incision into an avascular area was made with a clean scalpel. The electrode was then carefully inserted, making contact with the desired area with as little trauma as possible. Care was taken to avoid contamination with blood. The tissue was examined after the determination to detect evidence of any hemorrhage; results were discarded when this occurred. Readings were taken of the deeper, more central portions of the tumor as well as of the superficial portions. Since it has been shown that necrosis of tumor tissue alters tissue pH (21), the degree of macroscopic degeneration was noted and recorded. Several of the tumors or portions thereof were noticeably degenerated. All specimens were examined histologically by the hospital surgical pathologist, Dr. Alex Ragins.

To date, specimens from 110 surgical patients have been examined. Many of the specimens, for example, benign tumors of the uterus and breast, afforded the opportunity to make determinations of more than one distinct lesion in the same specimen. pH determinations of malignant tumors, benign tumors, normal tissues and a few inflammatory tissues were included in this study.

Included in the series of malignant tumors are: 20 carcinomas of the gastrointestinal tract, 10, 13 carcinomas of the breast, 1 carcinoma of the penis, 1 fibrosarcoma of the hip and 1 dermoid cyst of the ovary. Among the benign tumors were: uterine fibroids of 50 patients, 2 lipomas and 7 fibroadenomas and adenofibrosis of the breast. The small number of inflammatory tissues included appendix, gall bladder, cervix and sigmoid colon. The pH of normal tissues in specimens containing both neoplastic and normal areas were determined whenever possible. Incision into many areas of normal tissue caused bleeding from the tissue surface; these determinations were not recorded.

RESULTS

The pH of specimens of malignant tissue varied from 5.44 (carcinoma of the stomach) to 7.96 (carcinoma of the breast). Excluding those specimens which had undergone grossly marked degeneration and those in which preoperative irradiation had occurred, the pH range of these malignant tissues varied from 5.44 to 6.75.

A. Malignant Tissues.

Included in the 36 cases of malignant tumors studied were 14 cases in which we were able to determine the pH of normal areas as well as of malignant areas in the same specimen. In 12 of these cases, there was a difference in pH ranging from 0.17 to 1.15 with an average pH difference of 0.49, the lower pH representing that of the malignant tissue. In the remaining two cases, the pH of the uninvolved normal pectoral muscle was lower than that of the malignant area while the pH of the normal fatty and connective tissue was higher. These results are recorded below in Table I.

Specimen No. 94, representative of this group presented (Table I) is listed below in some detail in Table II.

Thirteen cases of carcinoma of the breast were examined. These included 6 cases which had not received preoperative irradiation and in which there was no gross evidence of necrosis at examination, 2 cases that had received preoperative irradiation and 5 cases in which there was gross evidence of necrosis. The following histological types were represented: medullary carcinoma, papillary adeno-

TABLE I: pH OF MALIGNANT TISSUES AS COMPARED TO pH OF NORMAL TISSUE IN THE SAME SPECIMEN (14 CASES)

	MALIGNANT TISSUE				NORMAL TISSUE				
Specimen and organ	Microscopic diagnosis	Number of readings	Range	Mean pH	Normal tissue	Number of readings	Range	Mean pH	Difference pH
No. 59 carcinoma, esophagus	Infiltrating squamous cell carcinoma	4	6.54–6.56	6.55	Muscularis	2	7.06–7.07	7.06	−.51
No. 73, carcinoma, esophagus	Anaplastic squamous cell carcinoma	6	6.32–6.43	6.39	"	2	6.77–6.78	6.77	−.38
No. 34, carcinoma, stomach	Adenocarcinoma antrum	8	6.37–6.72	6.55	"	2	7.19–7.20	7.19	−.64
No. 92, carcinoma, stomach	Papillary adenocarcinoma	6	5.94–6.24	6.10	"	4	6.59–6.72	6.65	−.55
No. 94, carcinoma, stomach	Infiltrating adenocarcinoma	10	6.19–6.32	6.24	"	6	6.52–6.70	6.62	−.38
No. 107, carcinoma, stomach	Scirrhus adenocarcinoma	8	5.98–6.18	6.06	"	4	6.48–6.55	6.52	−.46
No. 108, carcinoma, stomach	Infiltrating scirrhus carcinoma	6	5.93–6.08	6.01	"	4	6.39–6.42	6.41	−.40
No. 100, carcinoma, rectum	Infiltrating adenocarcinoma	8	6.39–6.48	6.43	"	4	6.64–6.67	6.66	−.23
No. 109, carcinoma, rectum	Infiltrating mucus-producing adenocarcinoma	8	5.61–5.82	5.72	"	4	6.06–6.10	6.09	−.37
No. 39, carcinoma, breast	Medullary carcinoma	6	6.72–6.75	6.73	Fatty tissue	2	6.90	6.90	−.17
No. 63, carcinoma, breast	Infiltrating papillary cystadeno-carcinoma	6	6.27–6.38	6.33	" "	2	6.80	6.80	+.47
No. 66, carcinoma, breast	Adenocarcinoma	6	5.86–6.11	5.95	Pectoral muscle	2	6.62	6.62	−.67
					Fatty tissue	2	7.10	7.10	−1.15
No. 103, carcinoma, breast	Infiltrating medullary carcinoma	10	6.01–6.17	6.09	Pectoral muscle	6	5.66–5.82	5.72	+.37
					Fatty tissue	6	6.31–6.36	6.34	−.25
No. 106, carcinoma, breast	Anaplastic medullary carcinoma	6	5.76–5.85	5.81	Pectoral muscle	4	5.24–5.30	5.27	+.54
					Fatty tissue	4	6.37–6.42	6.40	−.59

carcinoma, anaplastic carcinoma, carcinoma simplex, scirrhous carcinoma and adenocarcinoma. No correlation between histological type and tissue pH was determined. The influence of irradiation and that of degeneration on the pH of carcinoma of the breast is illustrated in Table III.

Single cases of carcinoma of the penis, teratodermoid of the ovary and fibrosarcomas were examined. The results of this miscellaneous group comprise Table IV below.

TABLE II: SPECIMEN NO. 94, EXTENSIVE INFILTRATING ADENOCARCINOMA OF THE STOMACH

10 minutes after removal. T26°C.

5 different areas of tumor: [1] and [2] through the mucosal surface into papillary portion of tumor:

[1] pH 6.32 6.32 [2] pH 6.24 6.24

[3], [4], and [5], through serosal surface into infiltrated portion of stomach wall:

[3] pH 6.19 6.20 [4] pH 6.21 6.21 [5] pH 6.24 6.23

Three areas of normal stomach wall—muscularis:

[1] pH 6.52 6.54 [2] pH 6.62 6.63 [3] pH 6.70 6.70

B. BENIGN TUMORS

Fifty cases of uterine fibromyomas were examined. Many of the uteri contained multiple fibroids and in approximately one-half of the cases, pH determinations were made of non-tumor bearing portions of the uterus as well as of the tumors. Two or more pH readings were taken in various

TABLE III: THIRTEEN CASES OF BREAST CARCINOMA

Specimen	No. of cases	No. of areas exam.	pH range	Mean pH
No preoperative irradiation. No degeneration	6	20	5.76–6.75	6.18
Preoperatively irradiated tumors	2	7	6.90–7.36	7.16
Grossly degenerated tumors	5	16	7.27–7.98	7.48

TABLE IV: MISCELLANEOUS GROUP OF MALIGNANT TUMORS

Specimen	No. of areas examined	pH range	Mean pH
Hornifying anaplastic squamous cell carcinoma of penis	3	6.57–6.62	6.59
Necrotic portion of above tumor	2	7.23–7.29	7.26
Teratodermoid ovary	2	6.82–6.86	6.84
Pleomorphic giant cell fibrosarcoma	3	6.17–6.39	6.25

parts of each tumor nodule. Many pH determinations were made of tumors which had undergone various types of degeneration. The minimal pH differences of this group are recorded in Table V.

TABLE V: FIFTY CASES, 151 DETERMINATIONS, OF UTERINE FIBROMYOMAS

Specimen	No. of areas examined	pH range	Mean pH
Normal uterus	21	6.57–7.17	6.94
Fibromyomas. No visible degeneration	112	6.11–7.31	6.76
Fibromyomas. Visibly degenerated	18	6.32–7.91	6.91

Seven cases of histologically benign breast tumors were examined, among which were 4 cases of adenoma and 3 of adenofibrosis. Table VI reveals

TABLE VI: SEVEN CASES OF BENIGN BREAST TUMORS

Specimen	No. of areas examined	pH range	Mean pH
Normal breast tissue	9	6.17–7.30	6.53
Adenoma of breast	13	6.32–7.18	6.82
Adenofibrosis of breast	9	6.38–7.18	6.81

that the mean pH of these tumors was higher than that of several normal areas of breast tissue in the same specimens.

Two cases of lipomas which were examined had a pH range of 6.77-7.21 with a mean pH of 6.96.

Experiments were carried out to determine the change in tissue pH in tumor specimens over prolonged periods following surgical removal. pH determinations were repeated in the same area at varying intervals. Representative of this group are 3 cases listed in Table VII.

TABLE VII: pH CHANGES IN TUMOR TISSUES OVER LONG PERIODS

(1) Medullary carcinoma of breast	30 minutes after removal	60 minutes after removal
	pH 6.75	pH 6.73
	6.75	6.73
(2) Fibrosarcoma of hip	30 minutes after removal	90 minutes after removal
	pH 6.39	pH 6.41
	6.39	6.41

(3) Fibromyoma of uterus

Time after removal	pH
10 minutes	6.90
20 "	6.89
30 "	6.88
40 "	6.88
50 "	6.88

DISCUSSION

The interpretation of pH determinations of tissues, especially those examined at varying intervals following removal from the body is at best somewhat difficult. First, we cannot evaluate with accuracy the rapidity and extent of change of tissue pH immediately after its blood supply has been severed and the tissue subsequently removed from the animal body. The formation of lactic acid and other acid metabolites in the tissues, the buffering capacity inherent in neoplastic tissue, the effect of the anaesthetic employed and the depth of anesthesia, the effect of parenteral glucose given during surgery, are important factors known to influence the pH of tumors. Further it is known that the pH of living tissue undergoes continuous variations and that the data obtained by various physicochemical measurements represent at best only a transient time differential. Another important factor, difficult to evaluate grossly and recognized by all workers as influencing tissue pH is the presence and variable degree of necrosis in different parts of the tumor. We know also that the electrode makes contact with many cells and with the intercellular fluid and not with one single cell. Obviously, we do not measure intracellular pH. Finally, the possibility that insertion of the electrode into the tissue may injure the smaller blood vessels and thus contaminate the electrode must be considered.

However, while it is impossible to eliminate certain of these potential sources of error, we feel that the careful technics employed, the accuracy of the apparatus used, as well as critical analysis of the results, enable us to draw some valid conclusions. Careful handling of the specimens following their surgical removal and careful insertion of a very small caliber electrode into the tissue avoids injury to small blood vessels and subsequent contamination of the electrode. When careful examination of the tissue and electrode following the determination revealed evidence of hemorrhage, the results were discarded. pH determinations of bloody fluid in other areas for comparison support this assumption. Loss of CO_2 from the tissue and consequent change of tissue pH is avoided as the capillary elec-

trode is deeply inserted into the tissue and the insulating electrode coating covers the upper portion of the tissue.

Using the fine capillary type of electrode, we noted an initial fall in pH of about 0.05 to 0.10 units during the first 1 to 2 minutes, this being followed with a gradual rise until the equilibrium value was reached. This primary fall is interpreted as being caused by cell injury and is of short duration.

Repeated experiments revealed that by withdrawing the electrode and carefully reinserting it into the tissue, pH readings could be duplicated. Therefore, the results are reproducible.

pH readings carried out at intervals in the same area of the tumor revealed that the voltage maintained a constant value for 90 minutes or longer, deviating at most 0.02 to 0.03 pH units. Confirming results of other investigators, we noted that necrotic portions of tumors gave consistently higher pH values than tumors which had not undergone degeneration. Of greatest significance in these experiments was the consistent pH difference between normal areas and malignant areas in the same specimen (in which cases almost all other factors are the same).

SUMMARY

Despite the many possible sources of error inherent in this approach to the study, we feel that they have been minimized by the careful technics and the accuracy of the apparatus employed. We have concluded that:

1. The pH of malignant tumors is lower than that of normal tissue in the same specimen.
2. The pH change of benign tumors varies. Some have a higher pH while others have a lower pH than that of the normal tissue in the same specimen.
3. Degenerative changes in malignant tissues increase the tissue pH.
4. Following irradiation, the pH of malignant tissue is increased.
5. The initial drift in pH to the acid side following insertion of the electrode appears to be less and its duration less in these specimens studied *in vitro* than in living tissue studied *in vivo*.

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Tumor Formation on Stem Fragments in Vitro*

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The formation of tumors in tissues of higher plants by the crown-gall organism, (*Phytomonas tumefaciens*), is influenced by several factors. First, the bacteria themselves must be virulent. Avirulent strains of the crown-gall organism will not produce a gall in susceptible tissue unless that tissue is also stimulated by a growth hormone (1, 10) or by products from a gall produced by fully virulent organisms (6). The tumefacient power of a strain of crown-gall organisms may be eliminated by the presence of certain amino-acids in the culture medium, the most potent of which is glycine (7, 11). The organism, even when fully virulent, has not been found capable of producing a gall unless introduced into wounded tissue. Very small wounds, involving only a few cells, were found to be sufficient to bring about gall formation, but when the wound was small, the gall was also small (Riker 1923). Introduction of bacteria directly into a plant cell was not followed by gall formation, at least in the tomato trichome (5). Not all higher plants respond with gall formation to inoculation with *Ph. tumefaciens*. Elliott (3) lists plants in 33 different botanical families, all dicotyledons, in which galls are formed in response to inoculation with this organism. There are, however, many species of plants, even among the dicotyledons, which appear to be immune to this organism, while, among monocotyledons, immunity is general.

Gall formation seems capable of occurring on practically every tissue of a susceptible plant from the apical growing point to the roots. The anatomical studies of Noël (1946) and other workers indicate that the epidermis, cortex, cambium and pith may all be stimulated to abnormal growth by the crown-gall organism.

That the formation of galls in susceptible plants is strongly influenced by temperature, has been demonstrated first by Riker (12) and later by Braun (2). Gall formation on tomato plants is completely inhibited if the plants are kept at a temperature of over 32° C., even though the bacteria remain alive and multiply at this temperature and the growth of the plants also is not inhibited.

Gall formation by *Ph. tumefaciens* on isolated plant tissue cultured *in vitro* seems not to have been studied to date. The use of isolated tissue fragments offers certain advantages over the use of whole plants so far as studies of tumor inception are concerned. The nutrition of isolated tissue fragments can be controlled, substances contributed by the plant as a whole can be excluded, while other substances, whose effect is to be tested, can be added in known concentrations to the nutrient medium. In this paper, a description will be given of the galls initiated by *Phytomonas tumefaciens* on isolated fragments of sunflower stem cultured *in vitro*. The effect of some nutritional and environmental factors on the formation of these galls will also be described.

MATERIALS AND METHODS

Sunflower stem tissue was obtained from plants of *Helianthus annuus* var. Russian giant grown in a greenhouse and used at the age of 4 weeks. Only the tissue of the first internode was used, being cut into fragments 4 mm. in length after having been freed from contaminants by the removal of the epidermis. These fragments were cultured at 25° C. on an agar medium containing 2 per cent sucrose and mineral salts as described by White and Braun (14). Five strains of *Ph. tumefaciens* were used having different tumefacient power when inoculated into the stem of the intact sunflower. The strains were cultured on sucrose mineral agar for 48 hours before use. In most instances the organisms were applied in aqueous suspensions, the viable counts of which could be ascertained by the method of colony counts.

EXPERIMENTAL

1. *Effect of strain of organism on formation of tumors on isolated stem fragments.* The 5 strains of *Phytomonas tumefaciens* used in these experiments differed among themselves both in cultural characteristics and in their power to produce tumors on intact sunflower plants. Strains BP and

* This work was done in part under an American Cancer Society grant recommended by the National Research Council Committee on Growth. The author is indebted to Miss E. Pieczur for technical assistance.

A_6 grew rapidly on sucrose mineral agar, strains B_2 and B_6 grew more slowly, and strain SP very slowly. In addition, strain B_2 was non-mucilaginous when grown on this medium. The differences in tumor size induced by these strains on the stems of intact sunflower plants are shown in Fig. 1. Strains BP and B_6 produced large tumors, strains A_6 and B_2 small ones, while strain SP produced no tumors.

These 5 strains were used to inoculate isolated fragments of sunflower stem. The fragments were cultured in vitro for 1 week before being used, as it had been shown (3) that such preliminary culture prevents, to a large extent, the passing of the organisms through the stem fragments onto the nutrient agar. It was desirable to avoid such penetration as the bacterial growth might interfere with the nutrition of the stem fragment. The pad of callus which had formed on the top of these fragments was removed, and the organisms were applied to the freshly cut surface by means of a loop. Two concentrations of organisms were used. The first, taken directly from the slope, contained about 50 times 10^9 viable organisms per loopful. The second, taken from a dilute suspension, contained about 50 organisms per loopful. After the stem fragments had been inoculated, they were incubated at 25° C. in continuous light for a period of 4 weeks. At the end of this time, the number of fragments with macroscopically visible tumors was obtained. The frequency of tumor formation in groups treated with different strains of *Phytomonas tumefaciens* is shown in Table I.

TABLE I: FREQUENCY OF TUMOR FORMATION ON ISOLATED SUNFLOWER STEM FRAGMENTS TREATED WITH DIFFERENT STRAINS OF *Phytomonas tumefaciens*.

Strain	Size of inoculum: 50×10^9 viable organisms	50 viable organisms	Total
BP	10/10*	17/20	27/30
B_6	10/10	16/20	26/30
B_2	0/10	0/20	0/30
A_6	0/10	0/20	0/30
SP	0/10	0/20	0/30

* First figure—number of fragments with tumors.
Second figure—number of fragments in group.

Strain SP, which lacked the power to produce tumors on intact sunflower stems, also proved incapable of stimulating tumor formation on isolated stem fragments. Strains A_6 and B_2 showed no sign of being able to produce tumors on the isolated fragments though they were capable of producing tumors of small size on intact sunflower plants. The highly tumefacient strains, BP and B_6, proved capable of producing tumors on isolated stem fragments, though the frequency of tumor formation by these strains was reduced when the size of the inoculum was cut down.

2. *Structure and appearance of tumors formed on isolated stem fragments.*—Fig. 2a shows the appearance of an uninoculated fragment of sunflower stem after 4 weeks culture on sucrose agar in light. A small callus pad is visible on the upper surface of the fragment. The base has increased in diameter more than the top, an effect due to the natural polarity of the stem. The fragment shown in Fig. 2b was inoculated on its freshly cut upper surface with 50 times 10^9 viable organisms of strain BP. A massive, symmetrical outgrowth of tumor tissue has resulted covering the whole upper surface of the fragment, pale green in color, thickest over the region of the cambium with a depression in the center suggesting that the cells of the pith had responded least to the stimulus. The stem fragment in Fig. 2c was treated on its upper cut surface with the small inoculum (about 50 viable organisms). No tumor formed at the site of inoculation but the organisms passed through the stem fragment and grew on the agar at its base. There resulted a basal tumor, more woody and compact than the apical tumor shown in Fig. 2b. A ridge of tumor tissue can also be seen growing out of the side of this fragment. Fig. 2d shows another stem fragment treated on its upper cut surface with about 50 viable crown-gall organisms. Here an apical tumor has developed, but, unlike the symmetrical tumor in Fig. 2b, the growth is confined to one side of the fragment. Here, also, the bacteria passed through the fragment and grew on the agar, resulting in the formation of a basal tumor on one side of the fragment. On the opposite side, a root developed, probably stimulated by growth hormones produced by the tumor tissue.

The stem fragments shown in Figs. 2e and f were both placed on their sides on the culture medium and inoculated with about 100 viable organisms on the upper side, midway between the cut ends. The fragment in Fig. 2e was inoculated directly on the surface of the cortical tissue which had been exposed by the removal of the epidermis. The latter process had presumably damaged a certain number of the cortical cells, none the less no tumor developed in the region to which the bacteria were applied. Instead, tumors grew at the two cut ends of the fragment, indicating that the bacteria had migrated in some way along the stem and initiated tumefaction in a region some distance removed from the point to which they had been applied. The fragment shown in Fig. 2f was punctured with a needle on its upper side before

the bacteria were applied. A tumor developed at the site of this puncture. In addition, tumors developed at the two ends of the fragment showing that, here also, bacterial migration had occurred.

Information about the internal structure of these tumors was obtained by fixing material in CRAF and examining sections stained either with safranin or haematoxylin. Fig. 3 shows a longitudinal section of a piece of untreated stem tissue after 4 weeks' culture *in vitro*. Figs. 3b, c and d depict similar sections of pieces bearing basal, apical and lateral tumors. The basal tumor shown in Fig. 3b resulted from cambial proliferation giving rise to masses of disorganized woody tissue. On one side of the tumor, a root arose which shows in the section growing out through the cortex. This basal type of tumor is shown in transverse section in Fig. 3i and can be compared with a transverse section through the same region of an uninoculated stem fragment (Fig. 3g). The tumor was a mixture of several cell types. The protoxylem was unchanged, nor could any signs of proliferation be observed in the pith. The bulk of the tumor consisted of newly formed xylem without large vessels or any internal organization, differing in appearance from the orderly tissue generated in the uninoculated stem fragments.

The small apical tumor shown in Fig. 3c was composed in part of cells which had arisen as a result of the multiplication of cortical and pith tissue. The core of the tumor was composed of woody tissue evidently generated by the cambial ring. Lateral tumors, such as those shown in Fig. 3d, appeared generally to arise from the cambium ring. They were composed for the most part of freshly generated xylem which appeared to have pushed through and ruptured the original cortical layer. Evidence of multiplication of pith cells within the protoxylem, which had been ruptured as a result, could also be seen in this section. A transverse section of one of these lateral tumors is shown in Fig. 3h. The section shows the proliferation of the cambium ring which has given rise to the woody tissue at the root of the tumor. The rest of the tumor seems to have arisen from proliferation of the cortical tissue. There can also be seen in this section a structure such as E. F. Smith referred to as a pseudostele, that is, an extraneous strand of vascular tissue lying in the cortex linked to a secondary tumor further along the stem.

Tumors formed on the physiologically basal or apical ends of the stem differed considerably in structure as can be seen from sections shown in Fig. 3e and f. The tumor shown in Fig. 3e resulted from the treatment of the apical end of a stem fragment with a large (approximately 50 x 10^9 viable organisms) inoculum of crown-gall bacteria. The resulting tumor was composed mostly of thin-walled parenchyma cells and had apparently arisen largely from the pith. The tumor in Fig. 3f resulted from the application of the same sized inoculum to the physiological base of the stem fragment. It resulted mostly from the proliferation of the cambium ring, was composed mainly of xylem surrounded by a sheath of parenchymatous cells evidently contributed by the cortex. The initiation of a root in the cortical tissue can also be seen in this section. The pith in this section evidently did not proliferate at all.

To obtain additional information about the vascularization of tumor tissue, a stem fragment with a large apical tumor, as shown in Fig 2b, was decolorized, cleared and stained for 24 hours in dilute alcoholic gentian violet. By this means, the vascular elements were stained while the other tissues remained colorless. A *camera lucida* drawing of this preparation as seen from above with the stem cut off across the base is shown Fig 4. It will be seen that some of the vascular strands fail to grow while others proliferated and branched freely, penetrating the tumor tissue and forming an almost continuous ring of vascular tissue. There is an obvious similarity in this behavior to the formation of the supporting stroma observed in animal tumors.

3. *Location of bacteria within tumors.* Six weeks old stem fragments bearing well developed tumors were rinsed in a small amount of sterile water. The water was then spread on nutrient agar which was incubated. In no instance were crown-gall bacteria recovered from these washings, from which it was concluded that viable bacteria were no longer present on the surface of the tumor. When, however, the tumors were removed, crushed, and then spread on nutrient agar, a heavy growth of bacteria was obtained. The organisms were apparently within the tumor tissue. Their exact location was not found. They were not visible in stained sections.

4. *Influence of wounding on tumor formation.* In this experiment, the bacteria were applied to the upper surface of the isolated stem fragments at different intervals of time after the latter had been isolated. The fully virulent strain BP was employed, and approximately 50 viable organisms were applied. In the first group, bacteria were applied as soon as the fragments had been isolated; in the second, three days later; and in the others,

one and two weeks after isolation. Frequency of tumor formation in these groups is shown in Table II.

TABLE II: TUMOR FORMATION ON ISOLATED FRAGMENTS OF SUNFLOWER STEM TREATED WITH *Ph. tumefaciens* AT DIFFERENT INTERVALS AFTER ISOLATION.

Time of application of bacteria	Proportion of fragments with tumors
At once	16/20
3 days	15/19
1 week	13/18
2 weeks	0/19

It appears that cells on the cut surface of the stem fragment remained in a condition to react with tumor formation to the application of *Ph. tumefaciens* for as long as one week after isolation of the fragment. After 2 weeks, the cells were no longer in a condition to react in this manner.

5. *Influence of light and temperature on tumor formation.* To determine whether light was essential for tumor formation, 2 groups, each containing 20 stem fragments, were inoculated on their freshly cut surfaces with about 50 times 10^9 viable crown-gall organisms, strain BP. Both groups were incubated at a temperature of 25° C., but one group was kept in continuous light and the other in continuous darkness. In addition, 2 further groups were prepared and incubated in darkness, 1 at a temperature of 25° C., another at a temperature of 33° C. After a growing period of 4 weeks, both groups were examined and the proportion of fragments bearing tumors ascertained as shown in Table III.

TABLE III: FREQUENCY OF TUMOR FORMATION IN GROUPS OF ISOLATED STEM FRAGMENTS CULTURED IN LIGHT OR DARKNESS AT 25 OR 33° C.

Treatment	No. with tumors	No. without tumors	Total
Continuous Dark	13	5	18
Continuous Light	14	4	18
Temperature 25°C	13	4	17
Temperature 33°C	0	20	20

Evidently, the formation of tumors on these isolated stem fragments was not influenced by the presence or absence of light. The experiment on the effect of temperature shows that tumor formation is inhibited entirely if isolated fragments are kept at a temperature of 33° C. This effect was not due to inhibition of growth of the stem fragments by the higher temperature. A comparison of the mean fresh weights of groups of stem fragments and of bacteria-free tumor tissue grown for 4 weeks at 25 and 33° C., revealed no significant differences in the amount of growth. Nor can it have been due to the effect of higher temperature on the bacteria. Strains BP and B_6 were maintained, with frequent transfers, for as long as 3 months, at 33° C., and tested every 2 weeks on isolated stem fragments. No indications were obtained of any loss of tumefacient power as a result of the high temperature at which the bacteria had been grown. Evidently, the actual process of tumefaction was interfered with by the high temperature, a phenomenon already described in intact plants by Riker (12) and Braun (2).

6. *Influence of nutrients on tumor formation.* It has already been shown that tumor formation on isolated sunflower stem fragments will take place provided that the fragment is supplied with sugar and mineral salts. Experiments were also performed to determine whether stem fragments which had been deprived of sucrose for various periods after inoculation with crown-gall organisms would still produce tumors when transferred to sucrose-containing medium. To ensure the elimination of stored carbohydrate, the fragments were incubated on sucrose-free agar for 5 days. After this period, they were deprived of the very slight pad of callus which they had formed and inoculated on the freshly cut surface with *Ph. tumefaciens* strain BP. They were transferred to sucrose agar in groups of 20 at different intervals after inoculation. After a further four weeks on sucrose agar, the different groups were examined for tumors. In the group which had been transferred to sucrose agar immediately after inoculation, 3 out of 20 fragments bore tumors. The other 2 groups, transferred 3 and 6 days after inoculation, showed no tumors at all. The third group, kept on sucrose-free agar after inoculation, also showed no tumor formation. Evidently, even the preliminary period of 5 days on sucrose-free agar was sufficient almost completely to prevent tumor formation on these isolated stem fragments. This effect was partly due to the fact that, even after 5 days on sucrose-free agar, many of the fragments became brown and were probably dead.

Experiments were also performed to determine whether isolated stem fragments retained their capacity to produce tumors when inoculated with *Ph. tumefaciens* after long periods of culture on sucrose mineral agar. Groups of 20 isolated stem fragments were cultured for 2, 4 and 6 weeks on sucrose agar and inoculated at the end of these periods on their freshly cut upper surfaces with a heavy inoculum of strain BP. There was no decrease in the number of tumors formed on stem fragments in the group cultured *in vitro* for 6

weeks. Evidently, although growth had practically ceased in these fragments, the factors necessary for tumor formation were still present in the tissues.

The position on the internode from which the stem fragment was taken had a pronounced effect both on the growth of the fragment and on its response to *Phytomonas tumefaciens*. Thus, in a group of stem fragments taken from the base of the first internode and inoculated after 5 days with strain BP, tumors developed on 13 out of 17 fragments. In a similar group taken from the top of the internode, 5 out of 16 fragments developed tumors. The average final fresh weight of fragments in the group from the base was 94 mg; that of fragments from the top was only 73 mgm. It seems possible that certain natural growth factors were concentrated toward the base of the internode and that these growth factors were responsible for the increased frequency of tumor formation on the fragments from the base.

SUMMARY AND CONCLUSIONS

Isolated fragments of sunflower stem cultured on sucrose agar were shown, in the experiments here described, to be capable of forming tumors when inoculated with a virulent strain of the crown-gall organism. They did not respond to those semi-virulent strains of *Ph. tumefaciens* which were capable of producing small tumors on the stems of intact sunflower plants. The tumors grew freely on the isolated stem fragments, although the unaltered tissue made little growth on the medium employed. They arose either on the physiological top or base of the fragments and were made up of cells contributed by the cortex, cambium, and, to a lesser extent, the pith. Fragments inoculated on the freshly exposed surface of the cortex developed tumors at their cut ends but not at the site of inoculation unless a deeper wound was made at this point. Evidence was seen in these fragments of the production of tumor strands similar to those described by Smith. Tumor formation was shown to be independent of light but strongly influenced by temperature, being completely inhibited when the inoculated fragments were maintained at 33° C., although neither the growth of the bacteria nor that of isolated stem fragments or bacteria-free tumor tissue was inhibited at this temperature. The surface of the stem fragments remained capable of forming tumors when inoculated within one week from the time of isolation. After this time, the healing process rendered the tissues unresponsive to the crown-gall organism. Tumor formation was very dependent on carbohydrate supply and could be almost completely suppressed by depriving isolated fragments of sugar for five days before inoculation. Prolonged culture of the fragments *in vitro* on sucrose agar did not prevent the formation of tumors on such fragments. Even fragments which had practically ceased growing after 6 weeks culture proved capable of responding to inoculation with tumor formation. The position of the fragment on the internode affected its growth and response to *Ph. tumefaciens*. Fragments from the base of the internode grew more rapidly and showed a higher frequency of tumor formation than did fragments from the top. This effect was probably due to polarity in the distribution of natural growth substances.

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DESCRIPTION OF FIGURE 1

FIG. 1.—Tumor production by strains of *Ph. tumefaciens* on stems of intact sunflower plants. (a) Avirulent strain SP. (b) Semi-virulent strain A_6. (c) Fully virulent strain BP. Natural size. (All photos by E. N. Mitchell).

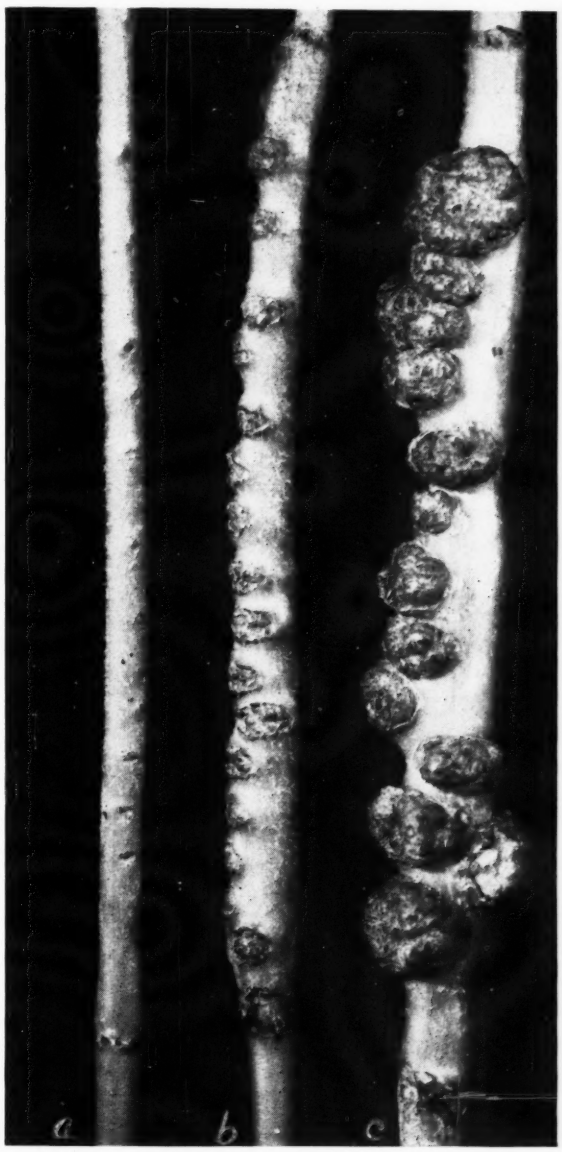

FIG. 1

DESCRIPTION OF FIGURE 2

FIG. 2.—Tumor production on isolated stem fragments of sunflower cultured *in vitro*. Mag. × 7. (a) Uninoculated fragment after four weeks culture. (b) Fragment inoculated on upper surface with about 50×10^9 viable crown-gall organisms, strain BP. (c) Basal tumor formed as a result of activity of bacteria growing on the agar. (d) Apical tumor on fragment inoculated on upper surface with about 50 viable organisms (strain BP). (e) Terminal tumors on fragment inoculated on its upper side midway between the ends. (f) Lateral and terminal tumors on fragment inoculated as (e) but punctured at point of inoculation.

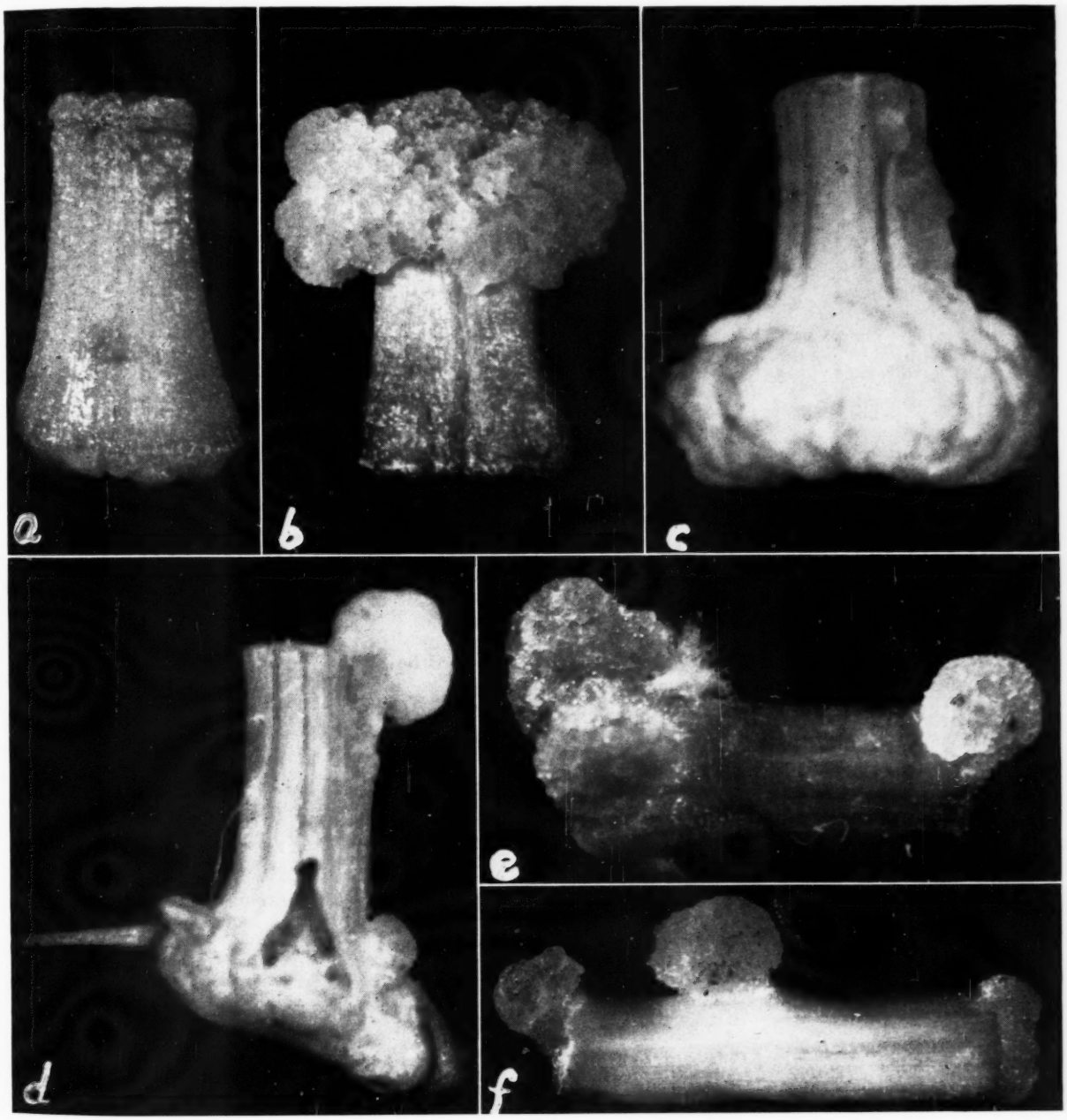

FIG. 2

DESCRIPTION OF FIGURE 3

FIG. 3.—Longitudinal sections of stem fragments grown *in vitro*. Mag. × 6.5. (a) Untreated. (b) Basal tumor showing root production. (c) Unilateral apical tumor. (d) Lateral tumor. (e) Tumor induced on physiological top of stem fragment composed mainly of parenchymatous tissue. Mag. × 6.5. (f) Tumor on physiological base of fragment composed of woody tissue generated by cambial ring. Mag. × 6.5. (g) Transverse section of base of uninoculated stem fragment. Mag. × 6.5. (h) Lateral tumor showing vascular connection and tumor strand (indicated by arrow). Mag. × 8. (i) Transverse section of base of inoculated fragments showing generalized proliferation. Mag. × 6.5.

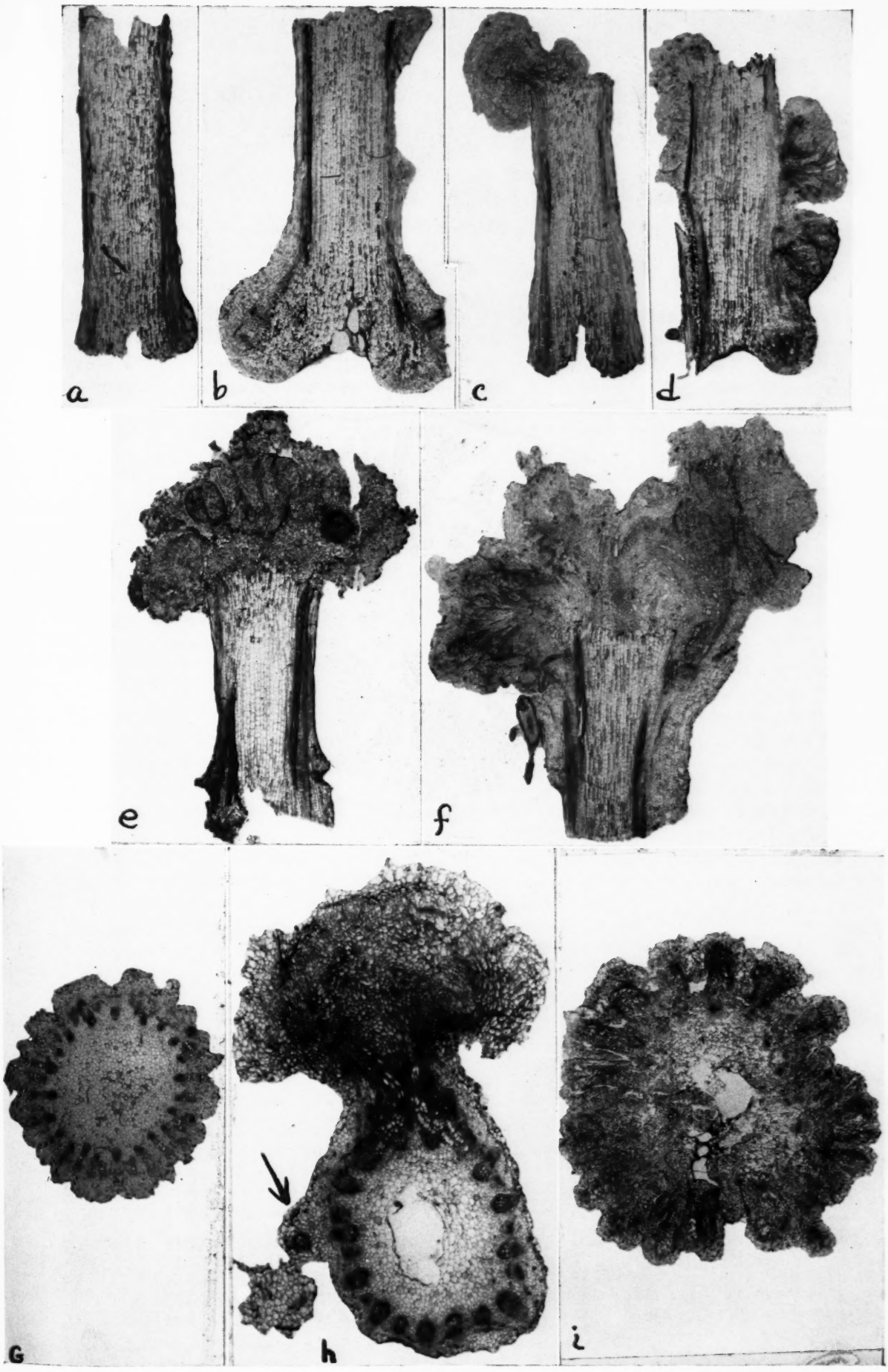

FIG. 3

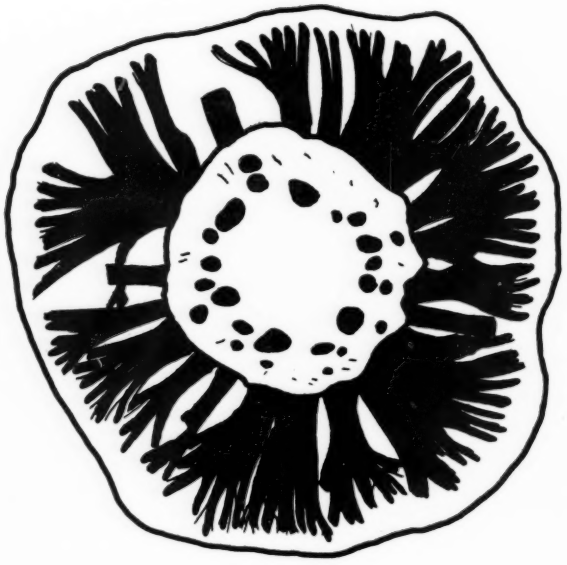

FIG. 4

DESCRIPTION OF FIGURE 4

FIG. 4.—*Camera lucida* drawing of apical tumor cleared and stained with gentian violet showing vascularization of tumor. Mag. × 26.

Effect of Beef Spleen Extract on Mitosis in the Small Intestine of the Mouse

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The present report of the effect of an aqueous alcohol-treated extract of beef spleen on mitosis in normal somatic tissues of normal mice is another of a number of studies to determine the mode of action of the beef spleen extract (4, 10, 11) which, in these laboratories, in collaboration with the Skin and Cancer Unit of the New York Postgraduate Medical School and Hospital, has brought about regression of basal cell epitheliomas (1, 2). In the present study mitotic activity in the epithelium of the crypts of Lieberkühn of the small intestine of the mouse was selected for comparative study. Diller (3) had established that a bacterial polysaccharide was injurious to dividing cells in the intestinal tract as well as to those in neoplastic tissue.

EXPERIMENTAL

Ten male dba mice, line 1, 6 to 8 weeks old, weighing 16±2 gm., obtained from the Roscoe B. Jackson Memorial Laboratory, were kept in a single wire mesh cage (12″ × 16″ × 8½″) on sawdust, and at a temperature of 21.5 to 23.5° C. Supplies of food and water were unlimited over the 16-day experimental period, during which time approximately 659 gm. of Rockland mouse pellets and 50 ounces of water were consumed by the 10 mice. The animals were weighed each day (Table I) on a balance accurate to 0.1 gm., since there is evidence that the nutritional status of the animal influences the condition of the intestinal epithelium (9).

The 5 experimental animals each received 0.1 ml. of the aqueous alcohol-treated extract of beef spleen (7) in the ventral abdominal region at intervals of 24 to 72 hours, spaced over the 16 day experimental period as shown in Table I. A total of 0.7 ml. of the extract, which contained 200-250 mgm. of solids per ml., was administered. The 5 control mice received identical treatment with an equal volume of Ringer's solution.

At 3:30 P.M. on the 16th day of the experiment the mice were killed by cutting the throat; 20 mm. of small intestine immediately distal to the pyloris was removed, fixed in Bouin's solution, sectioned at 6 μ and stained with hematoxylin and eosin.

Mitotic counts in longitudinal sections of the crypts of Lieberkühn were made (Mag. × 450) in an average of 10 fields each in 3 to 5 sections from different levels in the intestinal segment from each control and experimental mouse. The mitotic figures recorded included only those between the late prophase and early anaphase. A total of 239 fields and 1,160 mitotic figures were counted in the tissue from the experimental mice and 250 fields with 1,896 figures in those from the control animals.

RESULTS

From Figure 1 and the data presented in Table 2 it is apparent that under the conditions of this experiment an aqueous, alcohol-treated extract of beef spleen inhibited to a statistically significant

TABLE I: WEIGHT CHANGES IN DBA LINE 1 MICE DURING EXPERIMENTAL PERIOD

Mouse No.	Days 1*	2	3*	4	5*	6	8*	10*	11	12*	13	15	16*	Weight, gm. Av., S.D. (range)
							EXPERIMENTAL							
1	14.8	14.7	13.6	14.2	14.3	13.8	14.1	14.3	14.3	14.2	15	14.7	..	14.3±0.4 (13.6–15.0)
2	17.3	17.1	16.5	15.8	16.6	15.4	17.4	15.0	16.8	16.8	18.4	18.5	..	16.8±1.0 (15.4–18.5)
3	18.0	17.2	16.8	17.8	16.8	17.2	16.5	16.7	16.4	15.7	17.5	16.3	..	16.9±0.6 (15.7–18.0)
4	14.2	14.7	14.4	14.4	14.5	14.1	14.2	15.5	13.6	13.2	14.5	14.3	..	14.3±0.5 (13.2–15.5)
5	16.3	16.3	15.6	16.1	15.9	16.8	16.3	17.6	17.6	17.2	17.7	17.7	..	16.5±0.8 (15.6–17.7)
							CONTROL							
6	15.1	15.1	14.8	17.7	14.4	14	14.7	15.7	15.6	16.1	16.9	15.8	..	15.5±1.0 (14–17.7)
7	14.6	14.8	14.5	14.6	14.3	13.8	15.6	15.1	14.7	14.6	15.8	15.0	..	14.8±0.5 (13.8–15.8)
8	17.6	17.7	17.2	17.1	17.5	17.4	17.4	18.0	16.6	17.2	17.8	17.1	..	17.9±0.6 (16.6–18.0)
9	15.5	16.2	16.0	16.3	15.6	15.3	16.4	16.6	16.4	16.6	17.9	17.0	..	16.3±0.7 (15.3–17.9)
10	15.7	16.1	15.8	16.9	17.0	16.1	17.6	18.4	17.1	17.6	19.0	17.5	..	17.1±1.0 (15.7–19.0)

* Injections of Ringer's solution into control and of beef spleen extract into experimental mice.

extent the normal rate of mitosis in the epithelium of the crypts of Lieberkühn of normal dba mice.

TABLE II: MITOTIC FIGURES IN EPITHELIUM OF CRYPTS OF LIEBERKÜHN IN CONTROL AND EXPERIMENTAL DBA LINE 1 MICE RECEIVING SUBCUTANEOUS INJECTIONS OF AN AQUEOUS ALCOHOL-TREATED EXTRACT OF BEEF SPLEEN OVER A PERIOD OF 16 DAYS

Group	No. of mice	Total no. figures counted	Total no. fields counted	Average no. cells/field S.D., (range)
Experimental	5	1,160	239	4.8±2.0 (1–12)
Control	5	1,896	250	7.5±2.9 (1–15)

Total of 0.7 ml. of spleen extract or Ringer's solution given at 24 to 72 hour intervals in 0.1 ml. amounts.

Significance was determined by:

$$T = \frac{\bar{x}_1 - \bar{x}_2}{\sigma_D} \text{ where } \sigma_D = \sqrt{\frac{\sigma_1^2}{n-1} + \frac{\sigma_2^2}{n-1}}$$

$T = 13.5 \quad P < 0.01$

DISCUSSION

Both inhibitory and stimulatory materials have been obtained by Rohdenburg and Nagy (5, 6) from rabbit and human spleen as shown by the effects on reproductivity in the protozoa *Colpidium campylum*. The inhibiting action was in the initial acetone-soluble fraction, the stimulating material in the secondary aqueous extract from the tissue residue after the acetone extraction. Inhibitory substances were likewise obtained by the same method from benign dormant tumors. It was later shown by these workers (6) that transplanted rat sacromas continued to grow in the presence of the inhibiting materials.

Sugiura (8) found that the supernatant aqueous solutions from different rat organs had a depressant action on the growth capacity of mouse sarcoma 180 and the Flexner-Jobling rat carcinoma after storage in the fluid at 4 to 5° C., but that the most marked inhibition was with the fluid from macerated spleen. Similar results were obtained with the dbrB adenocarcinoma exposed to an alcohol-treated aqueous extract of beef spleen at 5° C. for 24 to 120 hours followed by transplantation of the tissue suspensions into dba mice (4). Concomitant cytologic study at the time of implantation revealed a cessation of mitosis *in vitro,* with degenerative changes appearing in the nuclei. Similar effects of the extract were noted on normal embryonic liver of mice (11).

The partial inhibition of mitosis *in vivo* in the crypts of Lieberkühn in mice receiving beef spleen extract as described in the present communication, and the cessation of mitosis in tumor (dbrB) tissue *in vitro* (4), somewhat parallels the findings of Diller (3) with the bacterial polysaccharide from *S. marcescens,* which inhibited mitosis not only in neoplastic, but also in normal somatic cells.

SUMMARY

An aqueous alcohol-treated extract of beef spleen injected subcutaneously over a period of 16 days (total, 0.7 ml.) into normal dba line 1 male mice resulted in a statistically significant decrease in the number of mitotic figures in the epithelium of the

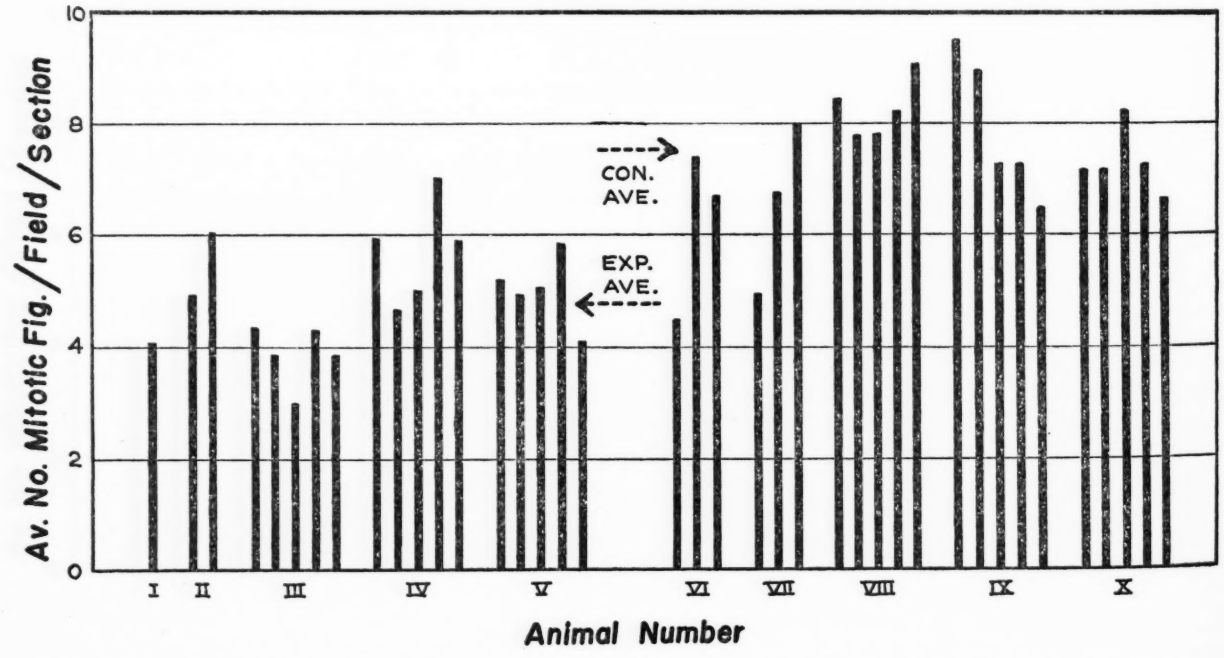

FIG. 1.—Effect of injections of beef spleen extract on mitotic activity in epithelium of crypts of Lieberkühn in dba, line 1, mice.

crypts of Lieberkühn indicating that the mitotic depressant action of the extract is not specific for or limited to neoplastic cells.

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Notes on the Electron Microscopy of Tissue Sections

I. Normal Tissue

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A century has been spent by microscopists and biologists in creating the present highly developed technic of preparing sections of biological material for the study of its structure by the use of the microscope. This work has been of the highest importance, not only in establishing a general knowledge of cell and tissue structure but equally so in the recognition and study of disease. However, it has become apparent that the rather low limits of the light microscope's resolution and consequently thereby its magnification, with a practical maximum of 1,200 to 2,000 diameters, constitute a frontier beyond which investigation cannot be carried. The microscopical differences between normal and malignant tissues have been well explored by the light microscope, within the limits of its capability. If knowledge is to be materially expanded in these fields, then an instrument of substantially higher resolution is required.

A few years ago the electron microscope was developed, and it awakened great hopes that it would open a new level of recognition in the study of the cell and the causes of its growth, its division and its multiplication. The instrument's resolution is 100 times greater than that of the light microscope and practical magnifications up to 100,000 diameters and higher are possible. Unfortunately the known sectioning technic by hand or microtome was found to be unsuitable because the sections so produced were many times too thick to allow sufficient penetration of the electron beam. Only coarse silhouettes ensued, revealing little or no detail of tissue structure. The difficulty involved can best be estimated from the work of Richards and Anderson (6, 7) who had to produce a thousand wedge sections by hand to find one a portion of which was suitable; in addition they had to employ an electron beam of the impractically high tension of 200,000 volts in order to obtain electron penetration. A practical technic of slicing tissue evenly and more thinly was necessary. In fact a thickness of approximately 1/10 of 1 micron, which is of the order of one fiftieth of the sections made by present methods, is demanded. Five years of electron microscope history passed before, in November 1943, O'Brien and McKinley of the University of Pittsburgh had the conception that a slicing knife moving at high speed might solve the problem (4). They did not show a machine or cross sections at the time but their idea was a definite lead. Attempts to construct a high speed microtome embodying this conception were undertaken in our Laboratories in the early spring of 1944 and today our knives, moving at speeds up to 1,100 feet per second, are capable of producing sections of the required thinness of the order of 1/10 of 1 micron (2). We are convinced that the hundreds of sections of normal and pathological tissue that were produced by our instrument have proven that the severe thinness requirements can be met and that electron micrographs possessing satisfactory detail can be produced. It is interesting to reflect upon the minute thickness of these sections by realizing that if they were standing on edge and viewed by a light microscope they would be at the limit of detectability, since their thickness dimensions are below the power of a microscope's resolution (3). We hope that the figures presented may demonstrate that the electron microscope can take over where the light microscope has reached its limit and that a new approach can be made to the study of tissue and its functions.

Another major difficulty presented itself and had to be solved before we could arrive at satisfactory electron microscope sections. The effects of coagulation and distortion of tissue protein produced by the known methods of fixation become very marked and objectionable under the much higher resolution of the electron microscope. Severe artifacts and shrinkage, not noticeable in light micrographs, are characteristic of the electron micrographs. A meth-

* The authors wish to express their full appreciation for the support of this work by a specific grant from the Lillia Babbitt Hyde Foundation.

od much more thorough and much more delicate is required to bring about that exact degree of fixation that is necessary to cut sections of the thickness of 1/10 of a micron and preserve their structural detail in dimensions so minute that they are far below the threshold of visibility in a light microscope. Such a fixation can result only from penetration of the fixative into the finest capillaries and details of protoplasmic structure as they are revealed by the electron microscope and which, at the same time, produces as little coagulation and shrinkage as possible. Proper fixation is most difficult to obtain when non-structured protoplasm occurs in the specimen, while the structural cell parts like the cellular and nuclear membranes, nucleoli, mitochondria and other secretory particles are much less affected by coagulation and therefore are subject to much less distortion. In addition and quite parallel to light microscope technic, electron microscopy requires and must develop in the future a technic for the differential staining of specific cell parts, which is a field practically unknown as yet.

Preparation of Tissue Sections for Electron Microscopy

For 2 years our work was limited to using normal animal and human tissue for the sectioning and study of normal tissue, and rat and mouse tumors for the study of neoplastic tissue. We have not been successful using routine fixatives like formalin, Zenker's solution, bichromate solutions, etc., because of the high degree of coagulation and shrinkage they seem to cause even if used in weak solutions. Osmic acid, on the other hand, produces a much lower degree of coagulation and in addition exhibits some selectivity for certain parts of the cell. The serious difficulty with the use of this fixative, however, is to obtain sufficient penetration throughout the fine detail of the tissue, and many attempts had to be made to solve this shortcoming. The first acceptable result was produced by the technic of perfusion, consisting of replacing the circulating blood in an anesthetized animal by Ringer's or Tyrode's solution and then obtaining fixation by forcing a 2 per cent osmic acid solution through the circulatory vessels. A fairly satisfactory degree of penetration into the tissue was obtained by this technic, and liver treated in this manner was suitable for high-speed slicing after the usual methods of dehydration and embedding either in paraffin or in camphor-napthalene. From the resulting tissue blocks good sections of the thinness of the order of 1/10 of a micron ensued. After they were selected, centered, and the screens punched by the help of the light microscope, their final electron micrographs showed good detail (1).

It is obvious that this method is not always suitable for the preparation of animal organs nor possible at all for the preparation of human material, and that other ways had to be found for an equally thorough penetration of the fixative. It should be pointed out again that this exacting and delicate fixation to preserve minute structural detail is not required to the same extent by the light microscope even for its highest possible magnifications. The much higher magnifications of the electron microscope, however, revealed such a degree of tissue distortion that the need for improved fixation technics was clearly indicated.

After much experimenting we were able to work out 2 methods or technics that appeared to give the most satisfactory results as to penetration and minimum of distortion. The one method depended on very gradual and slow fixation with solutions of osmic acid, followed by solutions of picric acid, both solutions increasing in a number of steps from highest dilution to solutions of approximately 1 per cent. The prerequisite of this method is the same as in perfusion, namely that blood be removed and washed out before fixation is attempted. The process is carried out in a vessel which is equipped with a slowly moving agitator and through which the solutions can be passed in a slow stream. While we have varied some of the details for many of our preparations, the following schedule may give an indication of the concentration of the chemicals used and the time of exposure to them:

Technic.—Thin tissue blocks placed in:

1. Tyrode's solution, 4 hours with agitation, several changes.
2. Tyrode's solution less glucose, 1 hour with agitation.
3. Osmium tetroxide .01 per cent,[1] 1 hour with agitation.
4. Osmium tetroxide .1 per cent,[1] 1 hour with agitation.
5. Washed with Tyrode's solution less glucose, ½ hour.
6. Picric acid, .017 per cent,[1] 1 hour with agitation.
7. Picric acid .17 per cent,[1] 1 hour with agitation.
8. Picric acid .85 per cent,[1] overnight.
9. Dehydrate in alcohols 5 per cent, 10 per cent, 20 per cent, 30 per cent, 50 per cent, 80 per cent, 95 per cent, 100 per cent (changed twice), xylol-alcohol 25–75 per cent, xylol-alcohol 50–50 per

[1] All solutions use Tyrode's solution less glucose for diluent.

cent, xylol 100 per cent (changed twice), paraffin embedding.

Although this fractional or gradual fixation brought a decided improvement, especially for tumor tissue, it was not able to fill quite as well the requirements for normal tissue. For the more recent part of our work we have developed and used a method which depends on a different conception. A combination of fixing agents was selected, which by themselves or in combination with each other cause little or no precipitation of proteins. However, since fixation at the end is more or less dependent on protein precipitation, it is meant here that the grain of the fixed or precipitated protein is so minute that in our observations it seems not to be visible or does not display the minimum of a disturbing factor in our electron micrographs. The agents were so selected that except for protein other tissue and cellular elements were fixed or retained with a minimum of shrinkage or distortion, and that the tissue was sufficiently hardened for slicing. An advantage of this fixation method is its ability to be used with equal success either on fresh tissue or tissue obtained from animals that have undergone perfusion with 10 per cent formalin while under anesthesia.

The agents selected were formalin, osmium tetroxide, potassium dichromate and chromic acid and, though they may not be able to cause sufficient cell fixation when used alone, they will, however, do so in combination. Roughly described, the fixation begins with 10 per cent neutral formalin which penetrates rapidly and which is a particularly effective fixative for protein. After thoroughly washing the tissue in running water, a mixed solution of osmium tetroxide and potassium dichromate is applied in a proportion giving equal penetration, and superior to either when used alone. Shrinkage that generally occurs in subsequent treatments like dehydration and embedding following a straight formalin fixation is, we have observed, very effectively prevented by this treatment. The final step without further washing is a short treatment with chromic acid solution, whose often drastic action is prevented here because most cellular elements have already been sufficiently fixed and hardened. After washing, the tissue is run through the standard method of dehydration and embedding, using, however, dioxane to replace the usual alcohol and xylol.[2]

[2] A special paper on the details of this technic is in preparation by its originator, Clifford E. Grey. We have named the method in our laboratories, "Grey's fixation."

DESCRIPTION OF FIGURES

The following figures were selected from our collection of hundreds of electron micrographs and they are limited in this paper to the types of tissue comprising liver, intestine and skin. Animal tissue was procured from freshly killed Rhesus monkeys, guinea pigs, albino rats and mice. Human tissue was procured from 5 New York hospitals and came directly from the operating tables. Our preparation of parts of the tissue selected by gross examination or by a low power microscope began immediately for animal tissue, and for human tissue in most cases within approximately 1 hour after extirpation. While en route, the human specimens were packed in iced containers and their case histories were fully available.

The series of these 3 micrographs is presented here to demonstrate not only the comparison of electron and light micrographs of the same area of a high speed microtome section, but Figs. 5B and 5C demonstrate that exposure of such a fragile thin section to the electron beam and the high vacuum of the instrument, even during the extended time necessary to shoot some 15 exposures for a montage, does not produce visible damage or distortion.

It should be stated that the normal human skin in Fig. 5 was derived from an area of an amputated female breast that was removed as far as possible from a carcinomatous tumor in this breast. It was pronounced to be normal skin by the pathological laboratory.

DISCUSSION

A deep and, we believe, far reaching difference is revealed between our electron micrographs and light micrographs of tissue sections. Detail and structure are revealed in the electron micrographs not only of material far below the threshold of visibility of the light microscope but equally of material that is quite within the range of this instrument, by the portraying of detail with materially intensified clearness and contrast.[7] We are con-

[7] It is a well known fact that the contrast in light micrographs can frequently be changed by the application of light filters. If contrast changes can be obtained within the narrow wave length range of the light spectrum, it can reasonably be expected that different contrasts will be obtained also when wave lengths that are as far apart from light as those corresponding to high voltage electrons are used. Detail fairly lacking in one type of micrograph may appear with great contrast in the other type. By this fundamental difference of operating in two widely separated parts of the electromagnetic spectrum, the light microscope and the electron microscope can theoretically be expected to supplement each other to great advantage in many instances.

vinced and have been able to observe with consistency that by such enhancement, morphological changes of tissue elements cannot fail to be intensified to a greater degree, so that slight changes or questionable conditions, as seen under the light microscope, are indicated more clearly and more distinctly under the electron microscope. We have not been able to find in the literature electron micrographs of tissue sections taken by other laboratories with the exception of the papers on insect cuticles by Richards and Anderson (6, 7) and by lack of extended previous experience our figures may present some difficulties of interpretation. Consequently we have included in this paper some light micrographs of the identical sections or sections from like areas from the same type of tissue in the hope that they may be helpful to bridge the gap existing between these two types of micrographs. It should be mentioned that our selection of material for electron microscopy was to a large extent guided by and dependent upon previous observations by the light microscope. Since our knife strikes the slowly advancing tissue block approximately 1,000 times a second and slices a thousand sections of the order of 1/10 of a micron thick, it can be reasoned that, after a second of actual cutting, the sections produced are within 100 microns of a given location that previously has been selected by light microscopy. From these sections, the most suitable ones are chosen by light microscopy before final electron micrographs are taken.

We would also like to mention that we became impressed with the advantage of the method of composite montages of electron micrographs. This method is carried out by scanning a whole complete tissue section first on the fluorescent screen of the electron microscope and then by a series of photographs in place of the fluorescent screen. When the micrographs thus obtained are assembled as in a picture puzzle and overlapping areas eliminated, a composite montage results which in the extent of the wide area covered is parallel to the large field of a low-power light micrograph, but excels it by its high degree of magnification. Our Figs. 3b and 5a are examples of this technic.

Two technics were worked out for the preparation of our tissue blocks which are of the approximate dimensions of 2 × 2 × 20 mm. They produced results equal or often superior to the known perfusion method carried out in living animals. These technics were calculated to accomplish fixation of specimen blocks with the least amount of noticeable precipitation and shrinkage and the highest amount of penetration. The types of tissue which we have prepared and sectioned by our methods were selected from the anatomical classes of liver, intestine and skin. We have not yet had the opportunity to study other classes of normal tissue nor to study exhaustively the types just mentioned and we wish therefore to present and merely report our present observations as notes on our work which we intend to follow by additional data in the not too distant future. We do not wish at this time to discuss the histological interpretation of the figures in greater detail than is given in the description accompanying them. We rather prefer, particularly in view of our following paper, "Neoplastic Tissue Sections," to speak of those observations which seem to present consistently some of the overall electron microscopic characteristics in the structure of normal cells and normal tissue, regardless of their anatomical location. These characteristics may be summarized as follows.

The outer cellular membranes as well as the nuclear membranes appear, in general, as solid, clean-cut, well defined lines. The granular particles of the cytoplasm can be clearly recognized in most cases and often a minor background of fibrillar structure consisting of very delicate and almost indistinct fibrils. The nuclei are well formed and the nuclear interior usually exhibits a very fine granular and uniform texture while the nucleoli appear to be much denser in structure. Intercellular tissue shows distinct fibrillar structure. Intercellular or intracellular round empty spaces having the appearance of "vacuoles" [8] appear occasionally to a moderate extent. A limited exception from these general characteristics was observed in some types of liver tissue inasmuch as its functional cells presented a wide variation in their different phases of storage and rest.

SUMMARY

Methods of sectioning, for the electron microscope, by the high speed microtome to the thickness of the order of 1/10 of 1 micron have been developed, as well as the auxiliary technics of fixing and embedding of specimens, to produce electron micrographs possessing satisfactory detail with a minimum of distortion by coagulation and shrinkage. These methods have been employed for the investigation by the electron microscope of sections of normal animal and human tissue, and hundreds of electron micrographs have been produced. Greater detail in general and some observations in particular have been presented by us in our electron micrographs which are not revealed by the light micro-

[8] The word "vacuoles" is used throughout the paper in a descriptive sense without connecting it with the routine definition.

scope, since dimensions are often involved which are below that instrument's threshold of resolution. The majority of our electron micrographs portray a quite consistent over-all picture in the appearance of cellular constituents. The outer and nuclear membranes appear as firm and well defined boundaries. The nuclei are generally well formed with fine granular texture and much denser nucleoli. The cytoplasm seems to be generally characterized by a coarser background with well defined and denser granular particles while the intercellular tissue shows submicroscopic fibrillar structure.

ACKNOWLEDGMENT

We wish to express our deep appreciation and thanks to Dr. Marion B. Sulzberger and Dr. Charles Sims of The New York Skin and Cancer Unit of the New York Post-Graduate Hospital as well as to Dr. Saul Kay of the pathological laboratories of the New York Post-Graduate Hospital for their great interest in our work and for providing us with specimens and full reports on the examination of these tissues.

We also express our heartiest thanks for most valuable assistance to Mr. Henry Green, Mr. Vincent Salines, Miss Marie W. Hartmann, Mr. Kenneth McCarty and Miss Helen Zarzycki, all members of the Research Laboratories of Interchemical Corporation.

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DESCRIPTION OF FIGURE 1

Fig. 1.—Comparison of sections of the same tissue block of normal guinea pig liver by light and electron micrographs. Prepared by perfusion[3] with osmium tetroxide and embedded in paraffin.

Fig. 1-A.—Light micrograph. Spencer microtome section about 5 microns thick taken at 1,000 diameters and optically enlarged to 2,000× magnification to enable easier comparison with Figure 1-B. Nuclei are clearly visible. Nuclear membrane fairly well outlined as well as nucleoli, The internal structure of the nuclei is hazy and somewhat spotty. The outlines of a few cells are discernible while the cytoplasm appears indistinct and somewhat vesicular. Mitochondria seem to be faintly indicated here and there.

Fig. 1-B.[4]—Electron micrograph, sectioned on our high speed microtome to about 1/10 micron thickness. Mag. × 3,000. Much more detail is revealed. The cells are clearly outlined and are distinct, separate units in the natural cell grouping. Cellular membranes are clearly and sharply outlined by distinct lines. The cytoplasm reveals structures of moderately dense small granules and a number of mitochondria which are too dense to show internal structure. The nuclei are uniformly shaped and their membranes sharply outlined. Their interiors consist of very fine and densely packed granular material evenly distributed throughout and contain 1 or sometimes 2 nucleoli. It is interesting to note that mitochondria and nucleoli are particularly well demonstrated with the electron microscope, probably for the reason that they absorb larger quantities of osmium tetroxide than the rest of the tissue. The high mass of the element osmium causes high density to the electron beam.

[3] Prepared by Dr. Albert Claude's laboratory at the Rockefeller Institute, New York.
[4] By courtesy of American Journal of Anatomy (2).

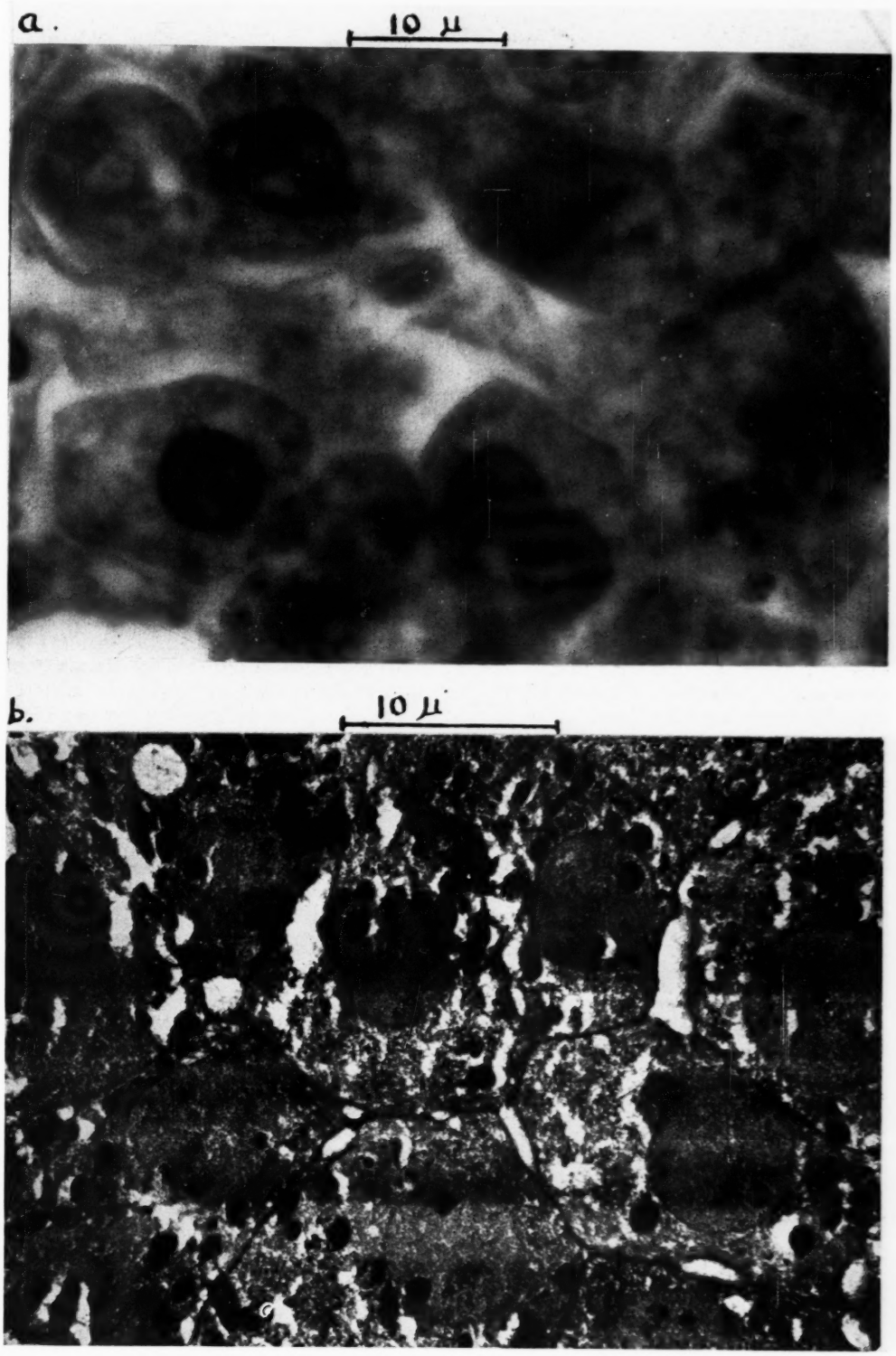

FIG. 1

DESCRIPTION OF FIGURE 2

FIG. 2.—An electron micrograph of a section of normal liver cells of Rhesus monkey prepared by perfusion with formalin,[5] followed by Grey's fixation, at 6,000× magnification. It presents a portion of two hepatic cells surrounded partly by connective tissue and intercellular channels which appear as empty spaces. The one cell contains a sectioned nucleus of dense internal structure with suspended heavy granular bodies, while the nucleus of the other cell must have been beyond the plane of the section. The cytoplasms of the two cells are filled with vesicles and granules. It is probable that the unfixed fat content of most of the globules has been dissolved out or has leaked out, since the thickness of the section is much less than the diameter of the globules. Membranes or shells of these globules are left behind, which we believe may not be true membranes but merely fixed cytoplasmic material in which the globules were suspended, or they may represent the outer layers of the fat globules more thoroughly fixed than the centers by osmic tetroxide. While some of the globules appear empty, others contain extremely fine material, probably consisting of fixed fat, of no apparent structure forming a uniform background in which smaller and larger granular material of various densities is suspended. It is interesting to compare this figure with those of Opie (5) who states that "The cytoplasm of liver cells consists in large part of vesicular bodies just within the range of microscopic visibility."

FIG. 2b.—Electron microscopic stereo of the same section at 2,300× magnification specifically presented here to show the great advantage of stereoscopic study. It portrays the spatial arrangement within the cell portions.

[5] Prepared by Dr. J. L. Melnick's laboratory at Yale Medical School.

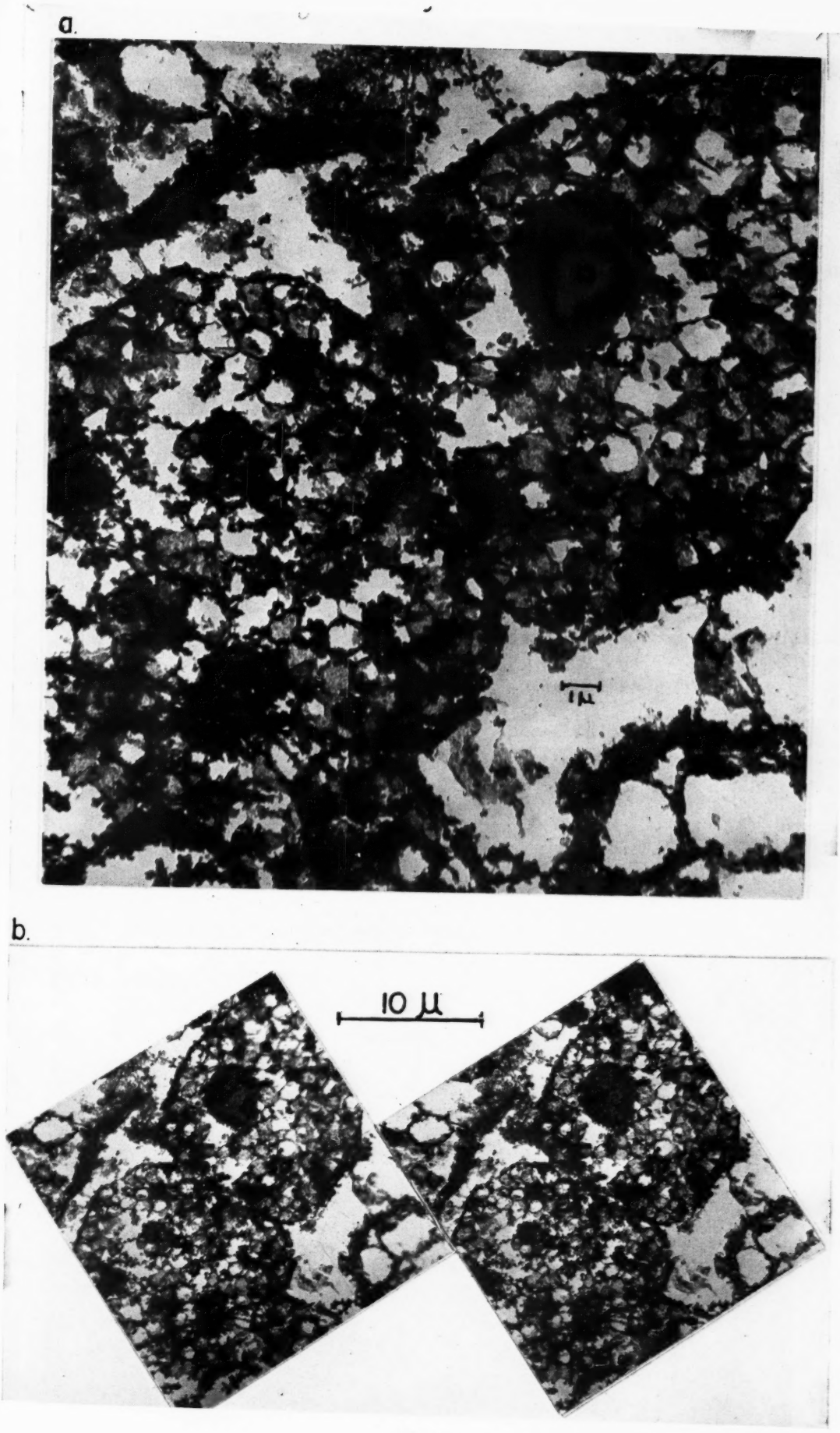

FIG. 2

DESCRIPTION OF FIGURE 3

FIG. 3.—Electron micrographs of sections of bile ducts of normal mouse liver at 5,000× magnification, fixed by Grey's fixation.

FIG. 3A is a transverse section and Fig. 3b is a longitudinal section. In Fig. 3a the bile duct, whose lumen measures approximately 10 microns in diameter, is fairly round but slightly ragged and open. The details of the five cells forming the encircling capillary wall are particularly clear in their natural position. The cytoplasm of these cells is a loosely arranged network, somewhat thicker near the edge of the cell membrane and around the nucleus. A number of mitochondria are present. The nuclei are finely granular and much more dense than the cytoplasm. They are sharply outlined though they appear hazy in areas where cytoplasmic material clings to the nuclear membrane. The cell membranes are clearly visible and show the cells lying closely packed with no intercellular spaces. The inner and outer vessel walls are continuous and well formed. The bile duct of Fig. 3-B seems to be partly collapsed. Its columnar cells are clearly shown with mitochondria in the cytoplasm and well formed nuclei which compose about one third of the cell. The outer vessel membrane is sharp and well defined.

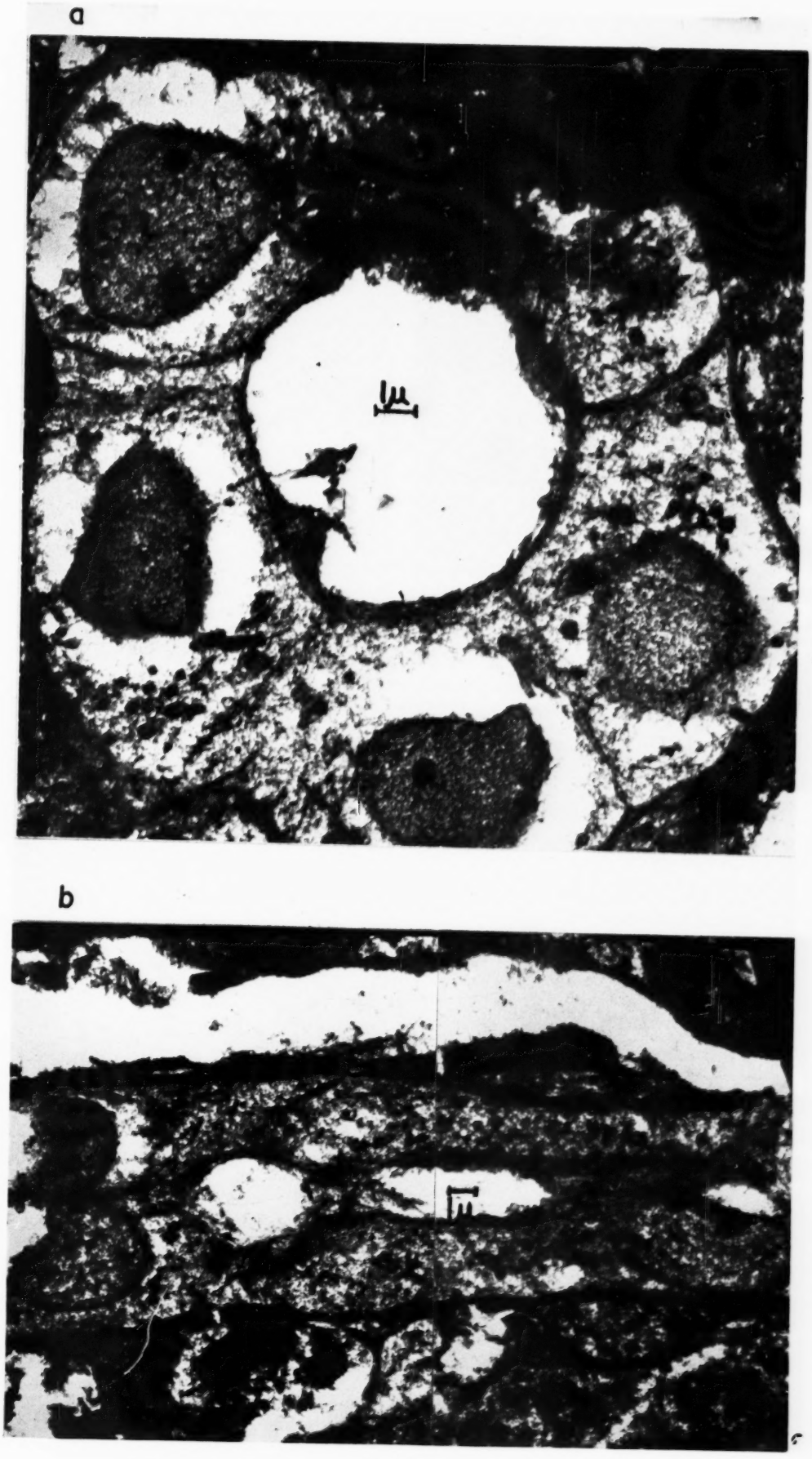

FIG. 3

DESCRIPTION OF FIGURE 4

FIG. 4.—Light and electron micrographs of intestinal tissue.

FIG. 4a.—Light micrograph at 200× magnification, fixed by formalin perfusion,[6] completed with Grey's fixative. It portrays the tip of a villus of Rhesus monkey intestine and shows particularly the appearance and positions of goblet cells. These mucus-secreting cells appear as white spots near the surface of the villus. The elongated shape of the nuclei appearing deep black is due to the lack of depth of focus. The epithelial cells of which they are part are not distinguishable.

FIG. 4b.—Electron micrograph of epithelial cells of mouse intestine transversely sectioned at 3,500× magnification and prepared with Grey's fixation. The well formed nuclei seem almost to fill the entire cell diameter of the closely packed columnar cells. The nuclear membranes are clear cut and the nuclear contents seem to consist of rather uniform granular material with some nucleoli in appearance.

FIG. 4c.—An electron micrograph of a section of normal Rhesus monkey intestinal villi at 4,500× magnification, prepared by perfusion with formalin followed by Grey's fixation. A single goblet cell, surrounded on the left side by epithelial cells is portrayed. The difference between the two types of cells is clearly shown. The nuclei of the epithelial cells are somewhat oblong with their membranes clearly shown. Some nucleoli can be recognized. The membrane of the goblet cell is well shown as well as the detail of its contents.

[6] Prepared by Dr. J. L. Melnick's laboratory at Yale Medical School.

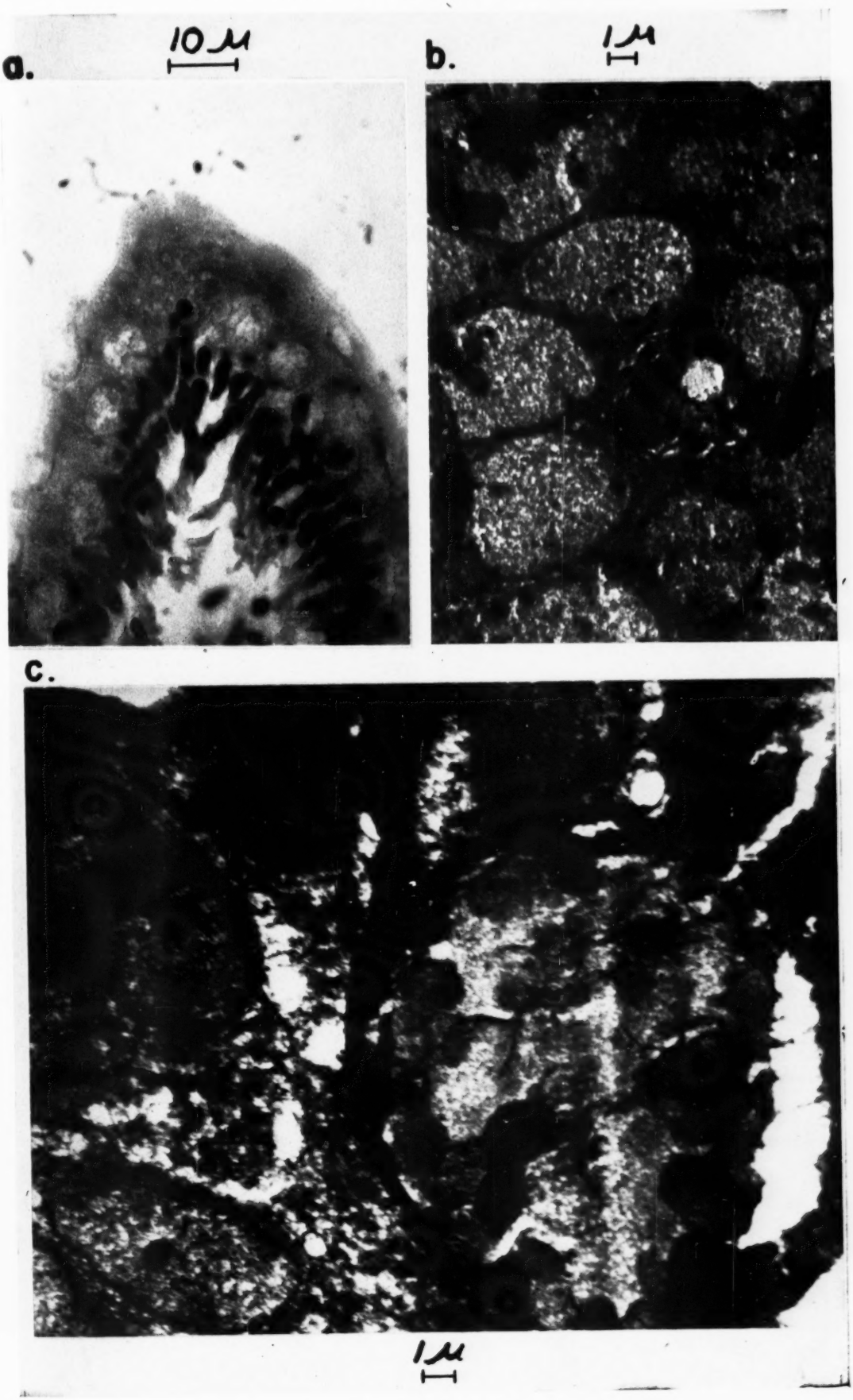

FIG. 4

DESCRIPTION OF FIGURE 5

FIG. 5 represents one and the same high speed microtome section of normal human breast skin, prepared with Grey's fixation.

FIG. 5b is a light micrograph at 600× taken before

FIG. 5a, a montage of some 15 electron micrographs at 1,800× magnification was taken, and

FIG. 5c is again a light micrograph at 600× magnification taken after the scanning of the electron micrograph montage in 5a.

The section represents the upper layer of cells in the stratum germinativum. Nuclei are either absent or they present the dry appearance characteristic of this part of the skin. The structure of the rather dry cytoplasm of the cells, the well defined cellular membranes, short intercellular bridges and enclosure of pigment granules are clearly portrayed in Fig. 5-A.

FIG. 5-D is an electron micrograph of a section of the same human breast skin at 5,000× magnification portraying the deeper layer of the epidermis. The Malpighian layer cells are connected to their adjacent cells by the typical spines in the form of bridges crossing the intercellular spaces. It is thought that the dense black strands in the lower half of the figure are the large, irregular pigment granules of the dermal chromatophores.

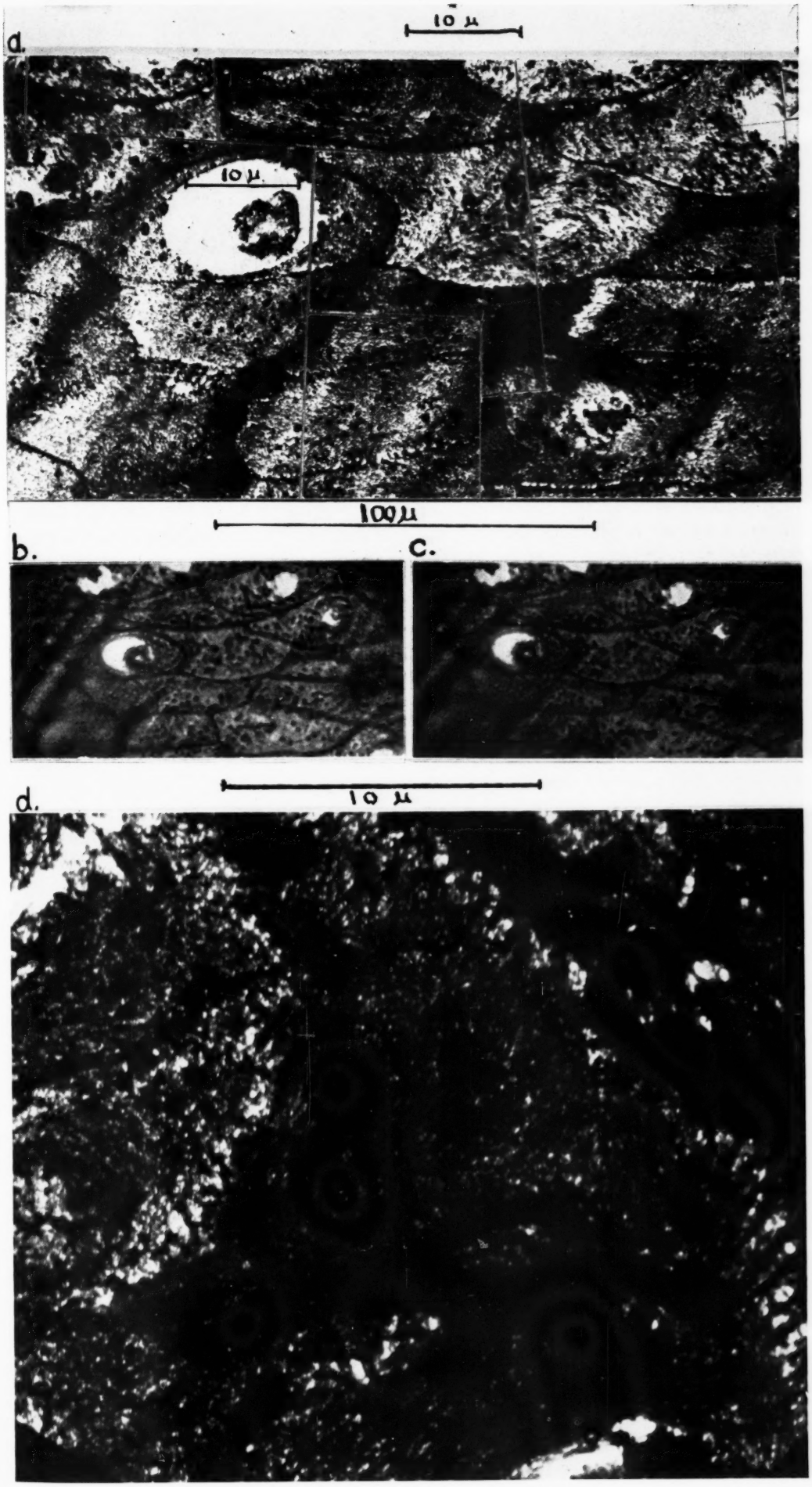

FIG. 5

Notes on the Electron Microscopy of Tissue Sections
II. Neoplastic Tissue*

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In the study of tissue sections with the electron microscope hundreds of micrographs have impressed us with some aspects characteristic of the over-all picture of cellular structure. We have observed the cells in our sections to exhibit such distinctive traits that we feel justified in listing some of these as indicative of normal tissue and some as pointing to neoplastic phenomena, regardless of the anatomical location of the tissue.

The electron micrographs of the majority of our sections of normal tissue portray an unmistakably consistent picture in the appearance of some of their cellular constituents. The membranes, either the outer or nuclear envelopes, are reproduced as firm, solid, clear-cut lines or boundaries. The nuclei are well formed, being round or oval in shape and their contents seem to be of a rather uniform, finely granular texture, while their nucleoli appear to be much denser in structure. The cytoplasm usually shows a coarser granular texture frequently with a fine fibrillar structure as a background consisting of delicate and often almost indistinct fibrils. Occasionally some round empty spaces having the appearance of "vacuoles" [1] appear in the connective tissue but only to a very moderate extent.

In contrast to such electron microscopic characteristics of normal cell structure, our sections of neoplastic cells seem to exhibit a somewhat aberrant picture. Greenstein emphasizes that tumors "tend chemically to resemble each other more than they do normal tissues or than normal tissues resemble each other" (5). It is not unreasonable to expect then that tumor cells would exhibit during their life cycle over-all characteristics in their structure generic to cancer that differ from those of normal cells. We did not wish to restrict ourselves to animal tumors induced by close inbreeding or the use of carcinogens whose limitations, determined by the present narrow state of knowledge, may constitute a very definite barrier in gaining a generic understanding of neoplastic phenomena. Such induced tumors may not be fully representative of human tumors arising from conditions unknown at the present time and so we have selected a major part of our tissue material from human origin. We believe that the hundreds of electron micrographs of neoplastic tissue which we have taken and studied in comparison with normal tissue sections may also justify us to list some common characteristics that impress us as specific to them, regardless of their location or source. We have selected the micrographs presented here from our large collection of electron micrographs of cancerous tissue sections in the hope that they may be best suited in the limited space available to present the subject and portray some of the aberrations that occur without and within the cells during cancerous development.

DESCRIPTION OF FIGURES

The material of our human tumor sections whose micrographs are presented here was selected by the pathological laboratories of the hospitals as well as by us and great care was taken not to include in our sections areas that displayed signs of necrosis. A list of the tumors as well as extracts from the reports of the hospitals on the tumors whose sections are presented here will be found in the Appendix.

In order to obtain a mental picture of different zones of development within the tumors, a method was adopted by which sections following progressively a diametrical path through the tumor were taken. By this method our sections generally start in the normal tissue adjacent to the tumor and progress through the apparent edge of the tumor proper, as probably its most active area, to the tumor center as generally the most indurated area. The micrographs presented here were obtained in this way and an attempt is made to present different

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* The observations described in this paper were first presented by invitation to a group of the faculty of Yale Medical School on September 24, 1946, in New Haven, Conn.

** The authors wish to express their full appreciation for the support of this work by a specific grant from the Lillia Babbitt Hyde Foundation.

[1] The word "vacuoles" is used throughout the paper in a descriptive sense without connecting it with the routine definition.

areas of development within the neoplastic growth particularly by a series of intestinal tumor sections.

DISCUSSION

We are convinced and have been able to observe that the indication of morphological changes of tissue elements is often intensified by the electron microscope. Slight changes from normal that may appear questionable as seen under the light microscope are in many cases indicated more clearly and more distinctly by the higher resolution of the electron microscope. We want to add to this sentence that it would not be unreasonable to expect that some histological characteristics connected with neoplasms would also be more clearly revealed.

Our experience has been limited to tumors of the intestine, skin, and breast, so far, but we believe that the consistent observations we have made in the study of hundreds of micrographs reveal sufficient generic characteristics of neoplastic tissue in contrast to normal tissue that an attempt can be made to catalogue and classify them. We are not aiming at an extended description of the morphological and histological detail revealed in our micrographs but rather are attempting to describe some of the generic manifestations by which our tumor sections seem to differ from sections of normal tissue of the same anatomical location. Thus, we have attempted to compare electron micrographs of sections of malignant epithelial tumors of the intestine to sections of the epithelial structure of normal intestine, or sections of malignant epithelioma to sections of normal skin. Unfortunately, due to the peculiar structure of breast tissue we have not been able so far to obtain satisfactory sections of normal human breast tissue though we encountered no difficulty in sectioning and portraying parts of malignant breast tumors. In consequence, our figures of breast carcinoma cannot be compared directly to the host tissue but we believe that notwithstanding they are important by their generic characteristics which are very similar to those portrayed in our electron micrographs of cancers of the skin and of intestine.

We are impressed by the consistency of a pattern that seems definitely to express a difference between normal and neoplastic tissue although we are fully aware that before these differences can be accepted as truly specific to cancer much more electron microscopic research must be done to ascertain that similar characteristics do not exist in some other types of non-cancerous tissue which we have not had the opportunity as yet to study. In this light we are presenting and reporting the observations we have made so far merely as notes on our work, which we intend to follow with additional data.

Our work is much too limited to give a clear electron microscopic description of neoplastic tissue, yet, as stated before, our electron micrographs of cancerous tissue impress us as exhibiting some definite characteristics which appear to be materially different from those of normal tissue. We do not know how many active neoplastic cells are portrayed in our micrographs since nobody as yet has been able to point out the specific difference between a malignant and a normal cell, nor do we know how many of the cells shown are normal cells that exhibit their reaction to nearby cancerous infiltration, yet all our cancerous sections seem, with surprising consistency, to follow a certain general trend. In cataloguing our observations we could sum it up by saying that signs of aberration in malignant tissue often begin with the appearance of "vacuoles" in the intercellular spaces of affected areas. It is emphasized here that the word "vacuole" is merely used in a descriptive sense without in any way connecting it with the routine definition of this term. It seems then, as aberration increases, that these vacuoles appear more numerous and also of larger size and are observed not only in connective tissue but also in the cytoplasm of some of the cells. It must be said here that vacuoles have been observed by us in our electron micrographs of normal tissue sections, yet they never seem to appear as numerous or as large. Their appearance in cancerous sections seems to be of a different order of activity. It is a possibility that they indicate a weakening or dissolving of tissue since one of the characteristics of tumor growth is its capacity to invade and break down adjacent normal tissue. It may be due, on the other hand, to lack of development of cells and supporting tissue, caused by too rapid a rate of growth. They may be the beginning of necrosis, though we have tried strictly to exclude necrotic portions of tumor. A phenomenon more important and generic than the appearance of large numbers of vacuoles in the cytoplasm seems to be a breaking or disappearing of the nuclear membranes. It is accompanied by a splitting or breaking of the nuclei into a number of fragments appearing as dense irregular masses. In great contrast to this appear the round or oval shapes of normal nuclei enveloped by clearly portrayed membranes and revealing a finely structured interior. This is the most characteristic observation that our electron micrographs of neoplastic tissue have enabled us to make since this phenomenon was persistenly portrayed no matter what cancerous tissue we have sectioned.

It should be added here that we also obtained the impression of the disappearance of nuclear membranes when we approached this problem from another angle, which concerned itself with the segregation of nuclei. By this work we were able easily to isolate normal cell nuclei in high purity and sufficient quantities, while we have not fully succeeded in duplicating this for nuclei of tumors. Most of their membranes seem to be destroyed or too fragile to stand up under the mechanical strain which this operation entails.

Another and often simultaneous phenomenon seems to be a similar breaking or disappearing of the outer cellular membranes, accompanied frequently by the appearance of fine fibrils of submicroscopic diameters. Often these fibrils, apparently coming from the extracellular tissue, seem to follow in their growth the outline of the disappearing membrane of the cell so that they frequently adopt the shape of a fibrillar shell. Often a complete filling of the cellular interior is revealed, with networks of fibrils replacing most or all of the cell and its surroundings. The presence of these fibrils in large numbers may explain in some parts the induration that so frequently occurs in tumors. Frequently the irregular dense masses of nuclear fragments are embedded in or encased by the network of these fibrils.

The diameter of fibrils as we have observed them is of the order of 100 A. in their first appearance of single fibrils. They seem to increase rapidly in number and dimensions and often the union of a number of single fibrils into ribbons or strands is observed. The fibrils themselves are constructed of regularly spaced segments and it should be noted here that judged by the dimensions and spacing of their segments they resemble the collagen fibrils described by Schmitt and collaborators (8). It is possible that the fibrils in our electron micrographs may be identical with the fine fibrils discussed by Howes (6) and it is interesting to note that Howes closes his paper by saying "It should be possible to investigate with the electron microscope the changes in the collagenous fibres and the reticulin that occur in spontaneous tumors."

In respect to this observation of an apparently progressing fibrillar growth it is interesting to reflect that, in all probability, the preponderant protein of these structures is collagen. The amino acid composition of collagen or rather gelatin, into which it converts so easily, is characterized by some rather exceptional proportions. One of them is the almost complete absence of tryptophane and another is the high percentage of arginine occurring in the order of an approximate ratio of 1 to 4 to 8 for histidine, lysine and arginine respectively (2), while normal muscle tissue has an approximate ratio of 1 to 4 to 3, and organs like liver, kidney and stomach, a ratio of 1 to 3 to 3 of these acids (1). Our many analyses of human and animal neoplastic tissue by microbiological assay [5] have very consistently disclosed a tryptophane level of half or less than that of normal muscle or organ tissue and have further disclosed a lysine-arginine ratio with arginine considerably in excess. Unfortunately, in human neoplasms not all of the tissue cell area consists of malignant cells (7) and the material we have analyzed must therefore be considered to be diluted or extended with normal tissue in different and varying degrees, so that our analytical results could only be expected to reflect a trend. Nevertheless the results, or the trend expressed by them, are sufficiently distinct to be interpreted as a support for the phenomenon observed in our electron micrographs that fibrillar replacement plays an important role in tumor development.

We were disappointed that no mitotic figures were found or portrayed in our electron micrographs since they are characteristic signs of cancer if investigated with the light microscope. A simple explanation, however, would present itself in the reflection that our sections for the electron microscope are of the average thickness of 1/10–3/10 of a micron while sections for the light microscope are 5 to 10 microns thick. A mitotic cell sliced to the latter thickness and in a favorable plane cannot fail to portray many of the mitotic bodies existing in it at that particular phase. However, the same cell sliced to the minute thickness of 1/10 of 1 micron, even if sliced in a favorable plane, could not portray whole mitotic bodies but only thin cross-sections of them, perhaps showing themselves as dots here and there that would be quite difficult to interpret. It is true that in many of our electron micrographs we have observed dots and lines here and there which we suspected to be sections of mitotic bodies but so far we have not been able clearly to demonstrate them as such. Also it must be reasoned that the fixations as used by us may happen to possess no selective absorption or affinity to chromosomes or mitotic bodies in general and that these thereby largely escape being portrayed by the photographic plate.

SUMMARY

High-speed microtome slicing of tissue into sections approximately 1/50 as thick as presently used

[5] To be published in a later paper.

sections for the light microscope, as well as new technics of fixation required by the high resolution of the electron microscope have been developed and employed for the investigation of neoplastic tissue. Some observations in particular are presented by our electron micrographs which we believe are not consistently revealed by the light microscope since often dimensions are involved below that instrument's threshold of resolution.

Signs of aberration observed in our electron micrographs of neoplastic tissue sections often seem to begin with the appearance of large numbers of vacuoles in intercellular tissue and cells. A characteristic aberration seems to be the breaking or disappearing of nuclear membranes and subsequent shrinking or breaking of the nuclear mass into dense fragments. A similar breaking of the outer cellular membranes frequently goes hand in hand, accompanied by the appearance of fibrils replacing them and often filling the cell interior.

In contrast, normal cells investigated by the same technics portray few vacuoles and solid, clear-cut boundaries for membranes. Their nuclei are well formed, round or oval shaped, and possess fine granular texture.

Every precaution has been taken to use only portions of tumors believed to be viable and we are convinced that some of the phenomena described are signs of neoplasia.

ACKNOWLEDGMENT

We wish to express our deep appreciation and thanks to the pathological laboratories of 4 New York hospitals. Dr. Marion B. Sulzberger and Dr. Charles Sims of The New York Skin and Cancer Unit of the New York Post-Graduate Hospital, Dr. Saul Kay of The New York Post-Graduate Hospital, Dr. Michael J. Horan of the House of Calvary and Dr. Milton B. Rosenblatt of New York City Hospital gave us invaluable support in our work by their great interest and help in providing us with tissue and its accompanying laboratory reports.

We also express our heartiest thanks for most valuable assistance to Mr. Henry Green, Mr. Vincent Salines, Miss Marie W. Hartmann, Miss Miriam Miller, Mr. Kenneth McCarty, Miss Gertrude Pfeiffer and Miss Helen Zarzycki, all members of the Research Laboratories of Interchemical Corporation.

APPENDIX

List of Tumors Used for Micrographs

Fig. 1	T–25
Fig. 2	T–25
Fig. 3	T–25
Fig. 4	C–5
Fig. 5	Animal tumor
Fig. 6	T–12
Fig. 7	T–7
Fig. 8	T–2
Fig. 9	T–2
Fig. 10	SK–1
Fig. 11	T–9

T–2.—Squamous cell carcinoma of skin
New York Post-Graduate Hospital
No. 22529
Female, age 53

T–7.—Biopsy specimen, adenocarcinoma of right breast
House of Calvary
Lab. No. 189
D.B. Female, age 57

T–9.—Mammary adenocarcinoma, cystic disease of the breast
New York Post-Graduate Hospital
No. 23030
Female, age 67

T–12.—Mammary carcinoma, secondary carcinoma of axillary lymph node
New York Post-Graduate Hospital
No. 23089
Female, age 80

T–25.—Carcinoma of transverse colon
New York Post-Graduate Hospital
No. 27114
Female, age 57

SK–1.—Superficial basal cell carcinoma
The New York Skin and Cancer Unit of the New York Post-Graduate Hospital
No. 16443
Female, age 40

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DESCRIPTION OF FIGURE 1

Fig. 1 presents an electron micrograph of a cross section of tissue of human intestine at 6,700× magnification, prepared with Grey's fixation.[3] Two goblet cells and a number of other epithelial cells are shown. This section originates from a part of the tissue that by gross examination is beyond the outer edge of the tumor.

[3] See Part I of this paper.

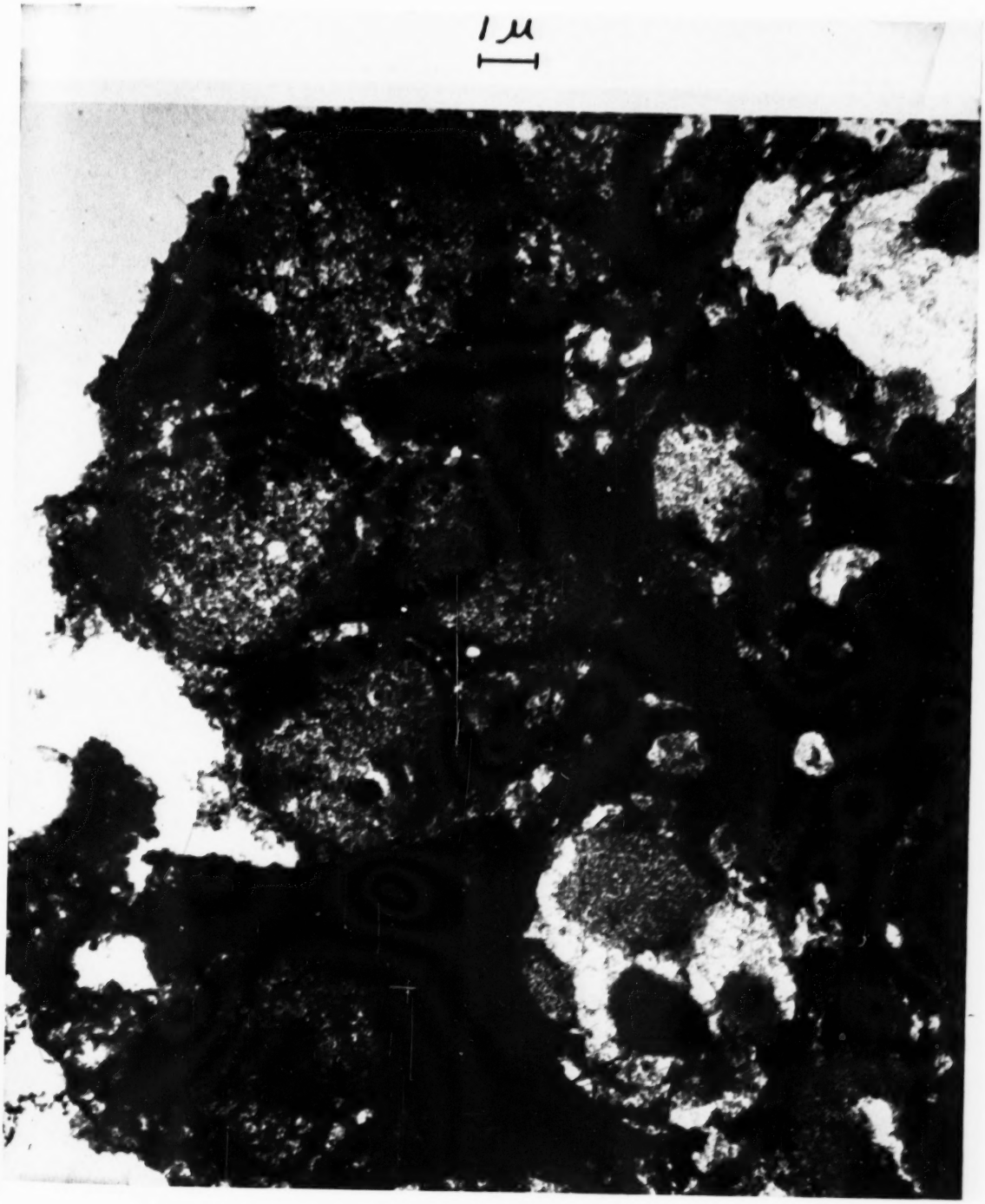

FIG 1

DESCRIPTION OF FIGURE 2

FIG. 2 is an electron micrograph of a section of the same human intestinal carcinoma as in the previous figure, at 6,700× magnification and prepared with Grey's fixation. Widespread appearance of vacuoles is strongly indicated within and without the cells, together with strongly represented fibrous structure. Some cells (a and b) appear to be foreign to this type of tissue and may be cancer cells. These cells differ from normal cells of the colon in the lack of uniformity in their over-all structure and in the presence of many vacuoles and fibrillar elements. Their membranes are not clear cut and particularly the nuclear membranes seem to be ragged and indistinct in most cases. Substantial evidence that vacuolization of the cytoplasm was present before fixation is the indentation of the nucleus in cell (a) Fig. 2.

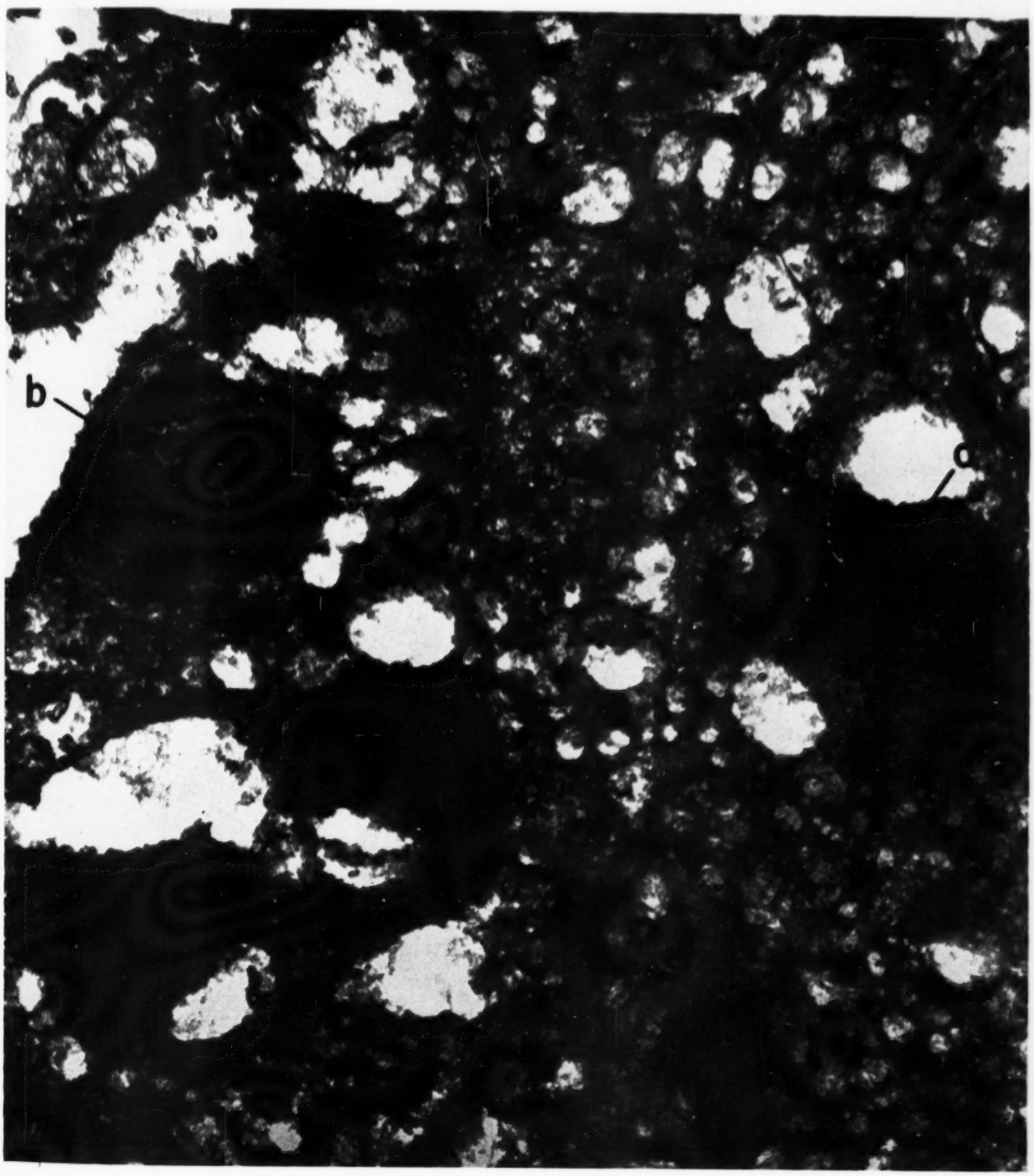

FIG. 2

DESCRIPTION OF FIGURE 3

FIG. 3 is an electron micrograph of the same tissue at 6,700× magnification and prepared with Grey's fixation. It seems to reveal greater differences from normal cells than the previous pictures, by showing still wider spread of vacuoles and fibrillar network. Three cells (a, b and c) are particularly shown whose cytoplasms are filled with vacuoles, whose cellular membranes as well as nuclear membranes exhibit ragged structure, and which seem to show distortion, possibly by pressure. The nucleus of the cell (b) at the left side and the cell (c) on top show severe spottiness.

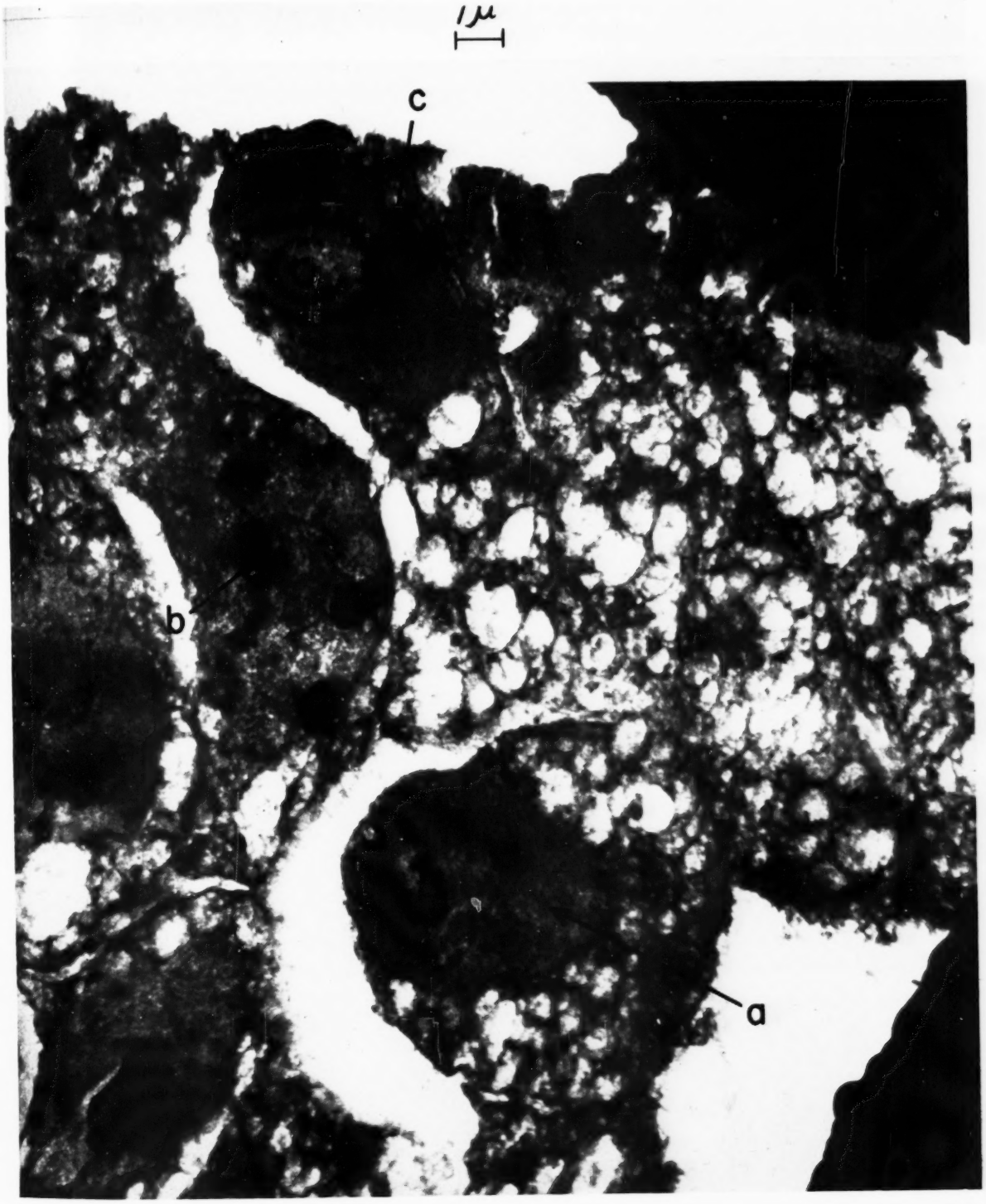

FIG. 3

DESCRIPTION OF FIGURE 4

FIG. 4a is an electron micrograph of a section of a human breast carcinoma prepared with 10 per cent neutral formalin in Tyrode's solution at 13,500 × magnification. It portrays a cancerous cell whose outer membrane has disappeared and has been replaced by a fibrillar envelope, part of which is clearly indicated. Fibrils have replaced cytoplasmic structure to a very large extent though some cytoplasmic bodies and granules are still present. Fibrils have bridged over and attached themselves to the nucleus which however seems to be still fairly intact though too dense to show interior structure. Fig. 4b of 2 stereoscopic electron micrographs of the same section should be advantageously viewed with a stereoscope to observe the three dimensional arrangement of the fibrils.

1 µ

a.

b.

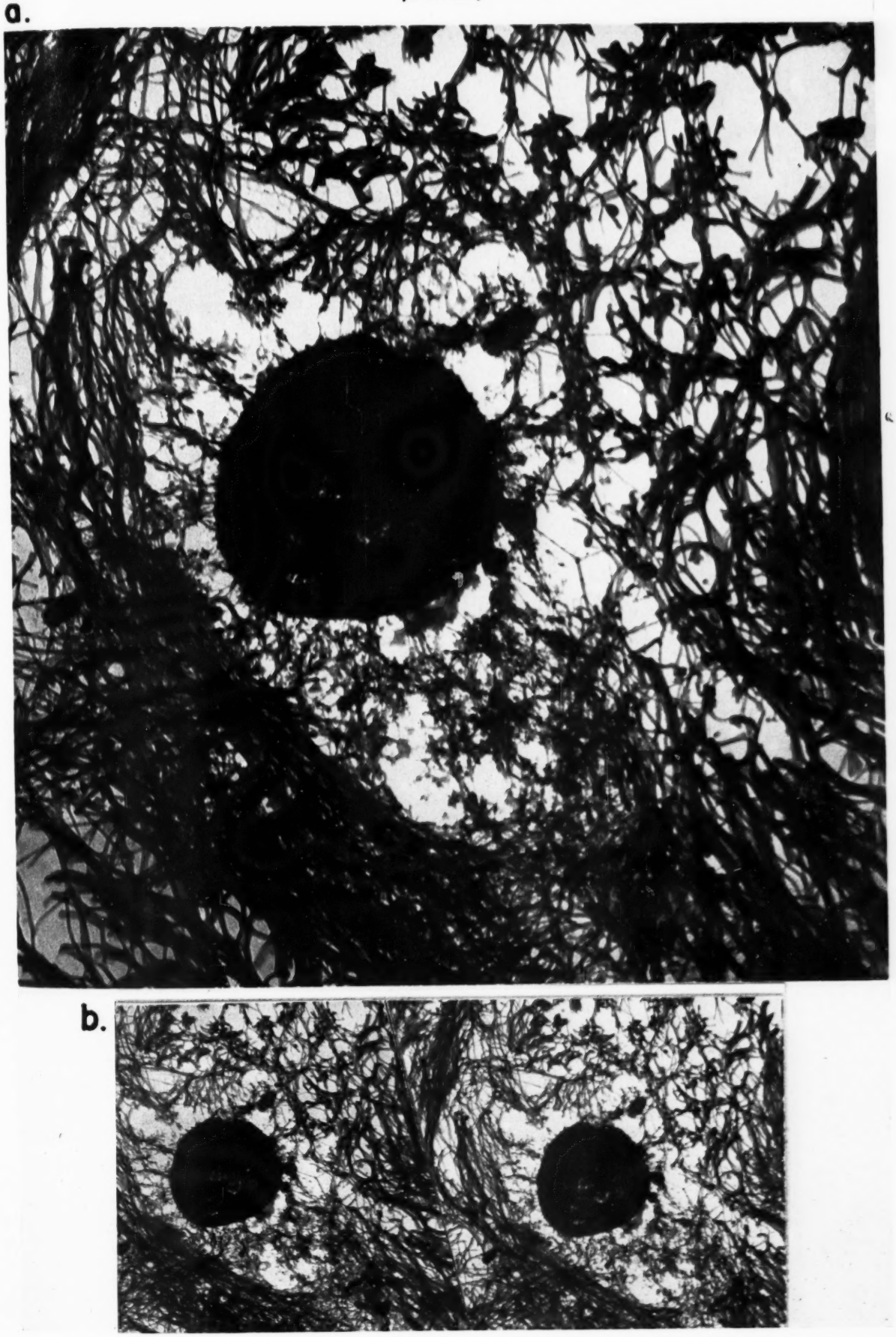

FIG. 4

DESCRIPTION OF FIGURE 5

FIG. 5 is an electron micrograph of a section of a mammary mouse tumor at 10,000× magnification prepared by gradual fixation with osmic acid and picric acid.[3] It appears to present a disintegrating cell surrounded by dense fibrous tissue. Both cellular and nuclear membranes have disappeared almost completely. The nuclear outline has become very ragged and the nucleus seems to be in the process of separating into clumps of dense masses. Occasional bodies resembling mitochondria and other granular material are still present.

[3] See Part I of this paper.

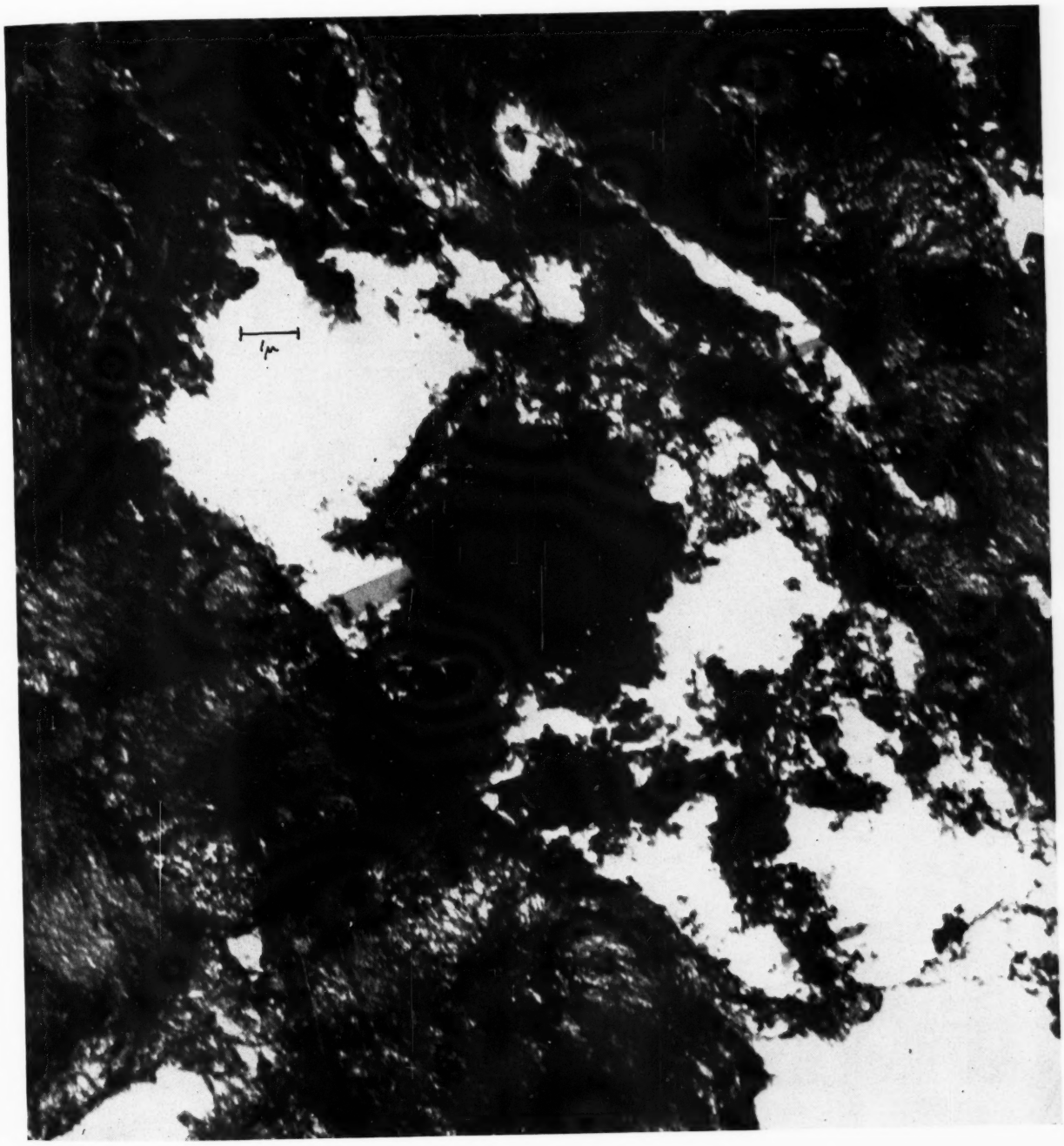

FIG 5

DESCRIPTION OF FIGURE 6

FIG. 6 is an electron micrograph of a section of a human carcinoma of the breast at 10,000× magnification. It represents in its main part a degenerated cell whose outer membrane has been almost entirely replaced by a fibrillar envelope strong enough to preserve the shape of the cell and parts of its contents. Large vacuoles seem to appear throughout the cell. Some opaque granular particles, perhaps mitochondria, are still visible as well as dense broken-up masses of fragments.

FIG. 6

DESCRIPTION OF FIGURE 7

FIG. 7 is an electron micrograph of a section of human mammary adenocarcinoma at 16,500 × magnification, prepared by gradual fixation with osmic and picric acids. The figure portrays what we believe to be 2 adjacent cells whose original enveloping membranes have been replaced by a network of extremely fine fibrils, thinner than in the previous figures. Fibrillar structure seems to be advancing from all sides into the cytoplasm, of which little is left except well preserved particles which may be mitochondria. The nucleus of the cell in the lower half of the picture has lost its membrane and its contents have been separated into clumps of heavy masses, while the nucleus of the upper cell seems to have disintegrated or have been outside the plane of the section, just as many sections of an egg could be made without striking its yolk.

FIG. 7

DESCRIPTION OF FIGURE 8

FIG. 8 is a montage of 5 electron micrographs of a section of a squamous cell epithelioma at 4,000× magnification and prepared with Bouin's fixing solution. The successful fixation of this section by Bouin's fluid proved to be an exception to the usual unsatisfactory results when using standard fixatives. This figure also shows a group of cells resembling cells of the stratum Malpighii. Nuclear degeneration is indicated in a number of cells (a, b and c) together with loss of nuclear membranes. In contrast, the two cells (d and e) at the extreme left of the micrograph seem to present a more normal appearance. The bridges between most of the cells seem to be well preserved and the tonofibrils in the cytoplasm of the cells are demonstrated.

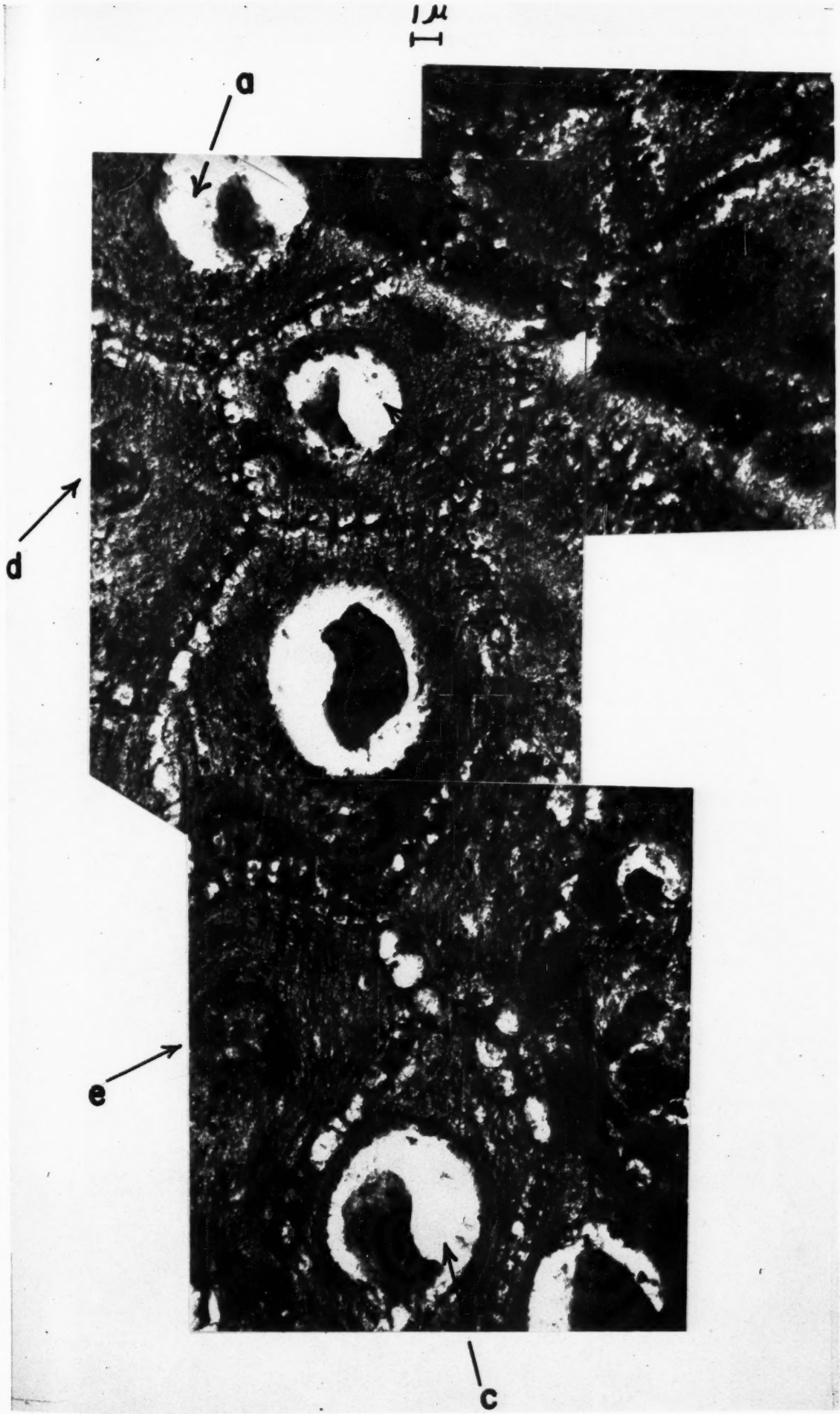

FIG. 8

DESCRIPTION OF FIGURE 9

FIG. 9 is an electron micrograph of a section of the same human epithelioma as Fig. 8 at 9,000× magnification, prepared with Bouin's fixation. The portrayed cell appears to be abnormal in the irregular formation of the nucleus and lack of a normal membrane.

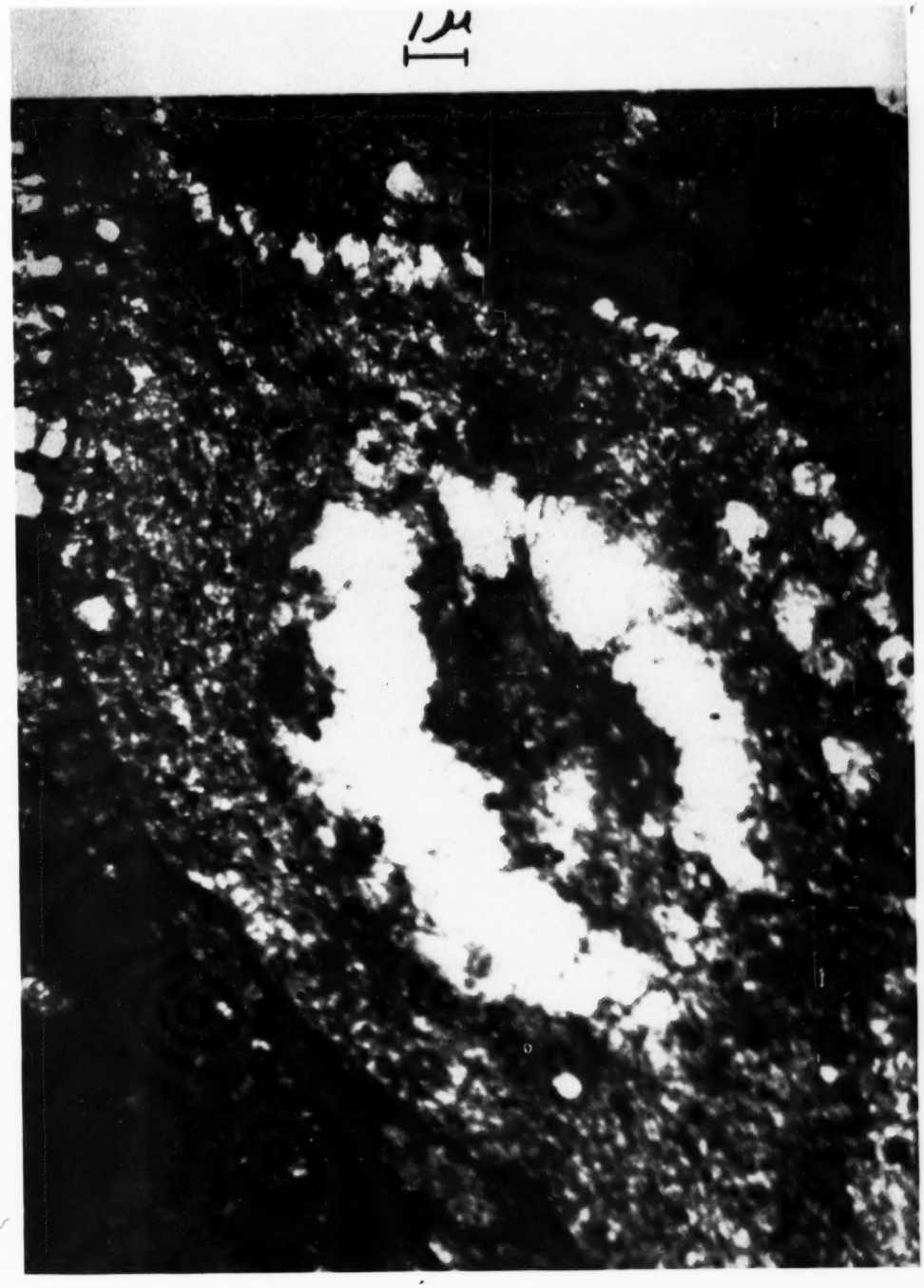

FIG. 9

DESCRIPTION OF FIGURE 10

FIG. 10 is an electron micrograph of a section of stratum Malpighii of the same human epithelioma as in Figs. 8 and 9. It is taken at 7,000× magnification and prepared with Grey's fixation. At least 4 cells can be recognized showing different stages of advanced nuclear disintegration. Fibrillar structures have replaced most of the cytoplasm of the cells in the upper half of the micrograph and their nuclei have broken up into a number of separate clumps. The intercellular bridges are gone, to a great extent, and cellular membranes are only partly preserved. The two cells in the lower part of the figure appear to be of normal development. The figure may represent the difference between disintegration of diseased cells and normal nuclear shrinkage of skin cells.

FIG. 10

DESCRIPTION OF FIGURE 11

FIG. 11 is a section of connective tissue of human carcinoma of the breast at 30,000× magnification. It has been prepared by gradual fixation with osmic and picric acids and has been intensified by chromium shadowing. It portrays at this magnification some of the fibrils that we have observed in many of the preceding pictures at lower magnification. Some of these fibrils are in single strands which appear to be the primary units, while others have joined to form strands of 2 or 3, producing thereby a flat band or ribbon effect. The beaded structure of the fibrils can be clearly observed whether they are single or in formations of double or triple strands. The diameter of single fibrils is of the order of 400–500A. The ends of the fibrils are broad or slightly rounded and cannot be observed in tipped or pointed form. It is difficult to decide whether the bulbing effect observed on some fibrils is a natural phenomenon or is due to a possible reaction of the protein of the fibrils with the fixing agents (3). The repeat distance of the segments seems to vary from 450 to 750A, which variation may be due to stretching or to a perspective effect. Submicroscopical spherical bodies which can be observed in this figure have already been reported by us (4).[4]

[4] A second report on submicroscopical spherical bodies will appear concurrently with this in "Experimental Medicine and Surgery."

FIG. 11

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FIG. 11

Plasma Changes in Fowls with a Transmissible Multiple Lymphoma

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(From the Biochemical Research Foundation, Newark, Delaware)

(Received for publication April 12, 1948)

Few studies have been made of the blood proteins of tumor-bearing animals. Sanders, Huddleson and Schaible (8) studied the pooled serum of chickens infected with various types of fowl leukosis, and Sharp, Taylor, Beard and Beard (9) the serum of papillomatous rabbits. Changes in the serum proteins were found by both groups of investigators.

In the present investigation the Tiselius method (11) was utilized to follow the effect of a rapidly growing and metastasizing transmissible multiple fowl lymphoma on the plasma proteins of individual chickens. A sharp decrease in albumin was found to accompany growth of the lymphoma. Although liver damage resulting from metastasis of the lymphoma seemed a plausible basis for this decrease, liver damage resulting from exposure to carbon tetrachloride vapors failed to produce a comparable change.

When spontaneous regression of the lymphoma occurred, there was an abrupt increase in gamma globulin. The injection of inactive lymphoma tissue, which produced no tumor growth, gave rise to plasma changes similar to those observed during regression; these changes were not produced by injection of normal liver tissue. Two chickens which proved immune to repeated implantation of active lymphoma tissue showed no significant change in plasma composition.

MATERIALS AND METHODS

Chickens. — University of Delaware line-bred White Rock chickens of the fourth generation were used for all experimental work. Families had been selected on the basis of high production factors and high viability throughout 14 weeks of age, and bred for use as broilers and efficient egg production. No cases of spontaneous lymphomatosis were observed during the entire experimental period in the fowls obtained from this source. The chickens were maintained on a diet of Purina mash for young chicks, scratch feed and pellets for older chickens, and water and grits *ad libitum.*

Tumor and tumor characteristics.—Fowl lyphoma tissue of strain RPL 16 was obtained from the U. S. Regional Poultry Research Laboratory, East Lansing, Michigan. The strain was carried through 26 transfers with no loss of virulence.

The characteristics of this transplantable strain of lymphoid tumor have been described in detail by Burmester and Prickett (2). The strain was derived from a case of spontaneously occurring lymphomatosis at the U. S. Regional Poultry Research Laboratory. It is characterized by multiple lymphoid tumors, uniformity of reaction, and high virulence. Burmester and Prickett (2) reported that all of the birds inoculated with the strain developed tumors, only about 6 per cent of which regressed, and the remainder of which proved rapidly fatal.

Transfer procedure.—A portion of the actively growing breast tumor and a portion of lymphomatous liver were removed aseptically and minced with fine scissors. The resultant tissue mash was diluted with twice its volume of sterile physiological saline and filtered through a double layer of sterile gauze. Inoculations were made into the right pectoral muscle with 0.5 ml. of the resultant cell suspension. Tumors and livers from a number of donors were macerated together whenever a large number of chickens were to be inoculated. Transfers were made every 7 days.

Electrophoretic analyses.—Electrophoretic analyses were made on plasma samples from individual chickens. Food was withheld for 24 hours before bleeding to eliminate suspended fats in the plasma except when the weakened condition of the animals made it inadvisable. Ordinarily 5 or 6 ml. of blood were drawn by cardiac puncture and centrifuged in tubes containing 0.8 mgm. of dry lithium oxalate as anticoagulant; when successive blood samples were drawn at short intervals, these quantities were halved. The plasma was diluted immediately with 3 (occasionally 2, 4, or 5) volumes of diethyl barbiturate buffer solution of pH 8.6, ionic strength 0.1, and dialyzed against two changes of the same buffer solution. Electrophoretic measurements were made either in the single section Tiselius cell or in the lower half of the double cell, at a potential gradient of about 12.0 V/cm. Photographs were taken by both the Svensson (10) and Longsworth (5) methods for 4 different periods of migration. Protein concentrations were computed from area measurements made on enlarged tracings of the four Svensson diagrams, corrected for the delta and epsilon effects, and expressed in refractive increments.

One hundred and fifty-five electrophoretic analyses were made on the plasmas of 108 chickens: 60 on the plasmas of 43 tumor-inoculated chickens, 49 on plasmas of 36 normal chickens, and the remainder on plasmas of chickens subjected to various types of treatment. In the graphical presentation of the data (Figs. 1 to 6) the concentrations of alpha and beta globulin and of fibrinogen and gamma globulin have been plotted together as the sums, both to simplify the graphs and to eliminate the effect of compensating errors resulting from poor resolution.

Plasma Protein Concentrations of Normal Chickens: Influence of Age and Sex

The plasma protein concentrations of healthy, uninoculated chickens at ages of 28, 61–2, 75, and 91 days are given by the solid black symbols in Fig. 1. It is evident that the composition of chicken plasma may vary between fairly wide limits at each of the age levels investigated. While the concentration range for the fraction alpha plus beta globulin was about the same at all 4 age levels, the mean for the fraction gamma globulin plus fibrinogen was somewhat lower at 28 days than later. The mean albumin concentration dropped appreciably between 28 and 61 days, but appeared to rise slightly between 61 and 91 days.

The sex differences found in mature chickens (6) were not apparent at 91 days (Fig. 1). The concentration ranges were wider for females, however, than for males (Fig. 1).

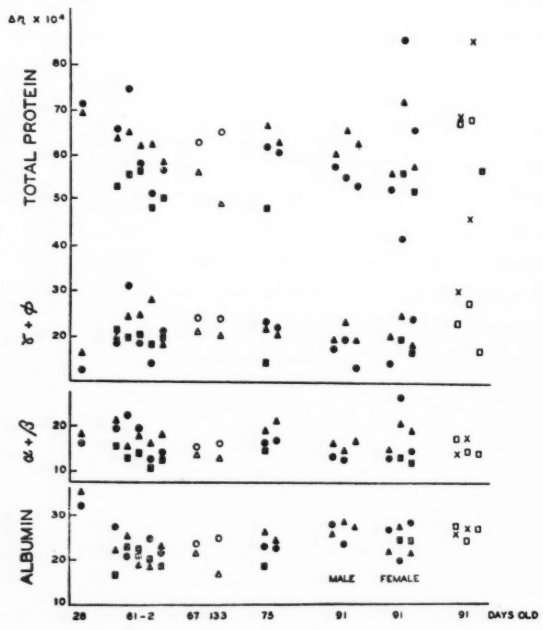

Fig. 1.—Plasma protein concentrations: ▲ ● ■, normal chickens; ○ △, tumor-resistant chickens; × □, chickens injected at the age of 52 days with minced normal liver tissue.

Plasma Changes Produced by Inoculation with Active Tumor Tissue

The effect of tumor growth on the protein composition of the plasma is shown best by the data on successive blood samples from individual chickens. Normal chickens inoculated with tumor cell suspension, 5 at the age of 61 days and 4 at the age of 75 days, were bled just before inoculation and twice weekly thereafter until death or sacrifice; 5 chickens 61 days old served as controls and were bled at the same times. In Figs. 2, 3, and 4 the plasma protein concentrations are plotted against the time in days following inoculation; points referring to the same chicken are connected by straight lines. The isolated points in the diagrams refer to single determinations on additional tumor-bearing chickens.

Tumor growth.—No significant change or trend in the protein composition of the plasma of normal chickens was disclosed as the result of successive bleedings (Fig. 2). On the other hand, the six

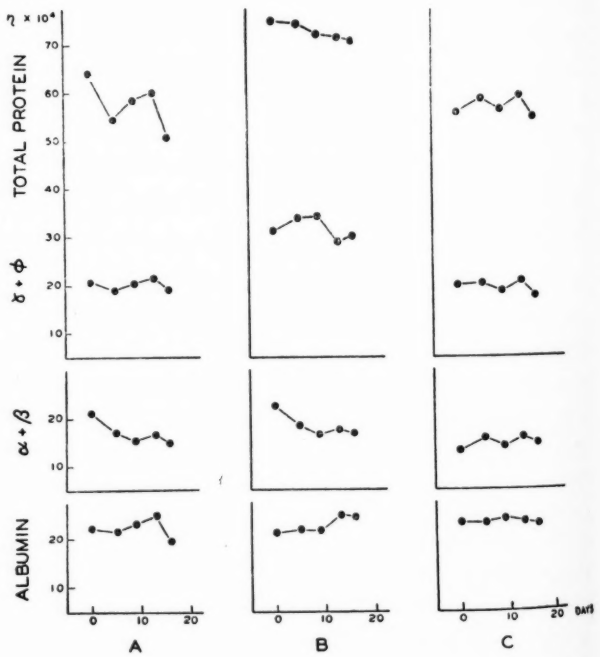

Fig. 2.—Effect of successive bleedings on the plasma protein concentrations of normal chickens.

chickens that developed tumors proving fatal within a week to 10 days of inoculation (Fig. 3) showed without exception an abrupt decrease in albumin concentration. Of the 3 inoculated chickens sur-

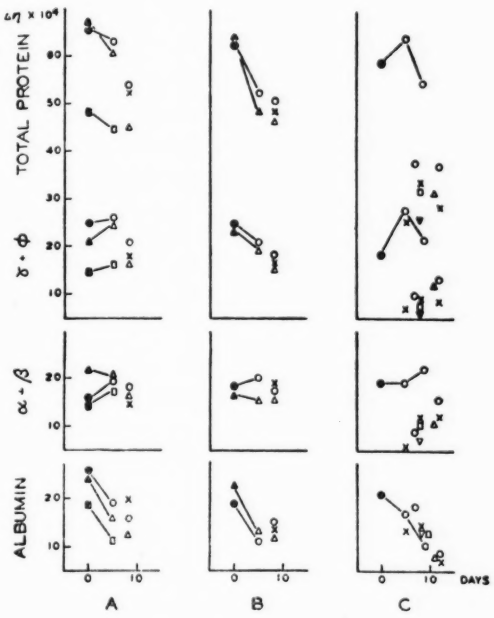

FIG. 3.—Effect of tumor growth on the plasma protein concentrations of chickens that died within 2 weeks of inoculation. Points referring to the same chicken are connected by straight lines. Solid black symbols, before inoculation; line symbols, after inoculation.

Figure	Symbol	Transfer	Age at Inoculation
A and B	●—○	26	61 days
A and B	▲—△ ■—□	26	75 days
A and B	⊙ × △	23	47 days
C	●—○—○	26	75 days
C	× , 5 days	11	28 days
C	○ , 7 days	7	82 days
C	▽ , 8 days	10	33 days
C	× □ , 8 days	12	34 days
C	△ , 11 days	2	45 days
C	○ × , 12 days	1	34 days

viving the 16-day period of observation (Fig. 4), two (A and B), which developed large tumors, also showed this decrease; the third (C), which developed only a small tumor, did not. The albumin concentration, therefore, was consistently reduced during rapid tumor growth. A second group of fowls that were inoculated at the somewhat younger age of 47 days, but for which the plasma composition prior to inoculation was not determined, also appeared to exhibit reduced concentrations of albumin 8 days after inoculation (Fig. 3, A and B, isolated points).

In still younger chickens inoculated at ages of 28, 33, or 34 days, and in 2 inoculated at 45 and 82 days respectively, rapid tumor growth was accompanied by low concentrations not only of albumin, but of the globulins as well (Fig. 3C, isolated points). While the albumin concentrations appeared to become progressively lower with time following inoculation, the concentrations of the globulins, particularly the alpha globulin, showed a tendency to rise, with the result that the alpha globulin fraction ultimately dominated the plasma pattern.

Tumor regression.—Of the 3 inoculated chickens that survived the 16 day period of observation (Fig. 4), two (C, which developed a small tumor,

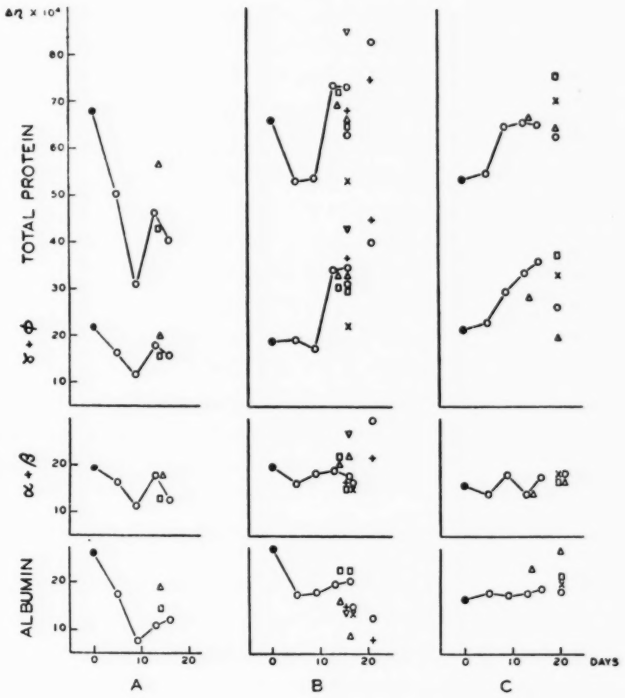

FIG. 4.—Effect of tumor growth on the plasma protein concentrations of chickens surviving inoculation 2 weeks or longer. Points referring to the same chicken are connected by straight lines. Solid black symbols, before inoculation; line symbols, after inoculation.

Figure	Symbol	Transfer	Age at Inoculation
A	●—○—○—○—○	26	61 days
B and C	●—○—○—○—○	26	75 days
A and B	□, 14 days	6	75 days
A and B	△ , 14 days	25	47 days
B	△ × ○ ▽ + □ , 16 days	3	53 days
B	+ ○ , 21 days	4	61 days
C	△ , 14 days	5	68 days
C	△ □ ○ × , 20 days	24	52 days

and B, which developed a large tumor) began to exhibit tumor regression about ten days after inoculation. Regression was in both instances accompanied by an abrupt increase in the concentration of gamma globulin. This sharp increase was not shown by the third chicken (Fig. 4A), the tumor of which remained large to the end.

A similar contrast in plasma composition resulting from tumor regression, as distinct from further

tumor development, was shown by 2 pairs of chickens, 1 inoculated at 47 days, the other at 75 days. Fourteen days after inoculation 1 member of each pair gave evidence of tumor growth, the other of tumor regression. The latter pair (Fig. 4B, 14 days) showed an elevated content of gamma globulin; the former pair (Fig. 4A, 14 days) did not.

The chickens represented by the isolated points in Fig. 4, C all exhibited early, rapid, and complete regression. The trend toward higher albumin and lower globulin concentrations in these chickens reflects the tendency for the plasma pattern to return to normal when regression is complete.

The plasma composition of the chickens in which delayed tumor regression occurred at 16 and 21 days is plotted in Fig. 4, B. While the changes accompanying this condition were similar in direction to those accompanying earlier regression, they were in general more profound. Elevation of the globulin levels was not restricted to the gamma globulin, but embraced the alpha plus beta globulin fraction as well.

Tumor-resistant chickens.—Two chickens proved resistant to repeated inoculations with active tumor tissue. Plasma obtained on the 67th day, following inoculations on the 42nd and 55th days, showed no departure from the normal (Fig. 1, ○ △, 67 days). A third inoculation on the 75th day, a fourth inoculation on the 98th day 4 days after x-irradiation with 300 r, and a fifth inoculation on the 116th day immediately after x-irradiation with 600 r, caused in one of the chickens no change of plasma composition with respect to the earlier bleeding, but in the other a reduction in albumin (Fig. 1, ○ △, 133 days).

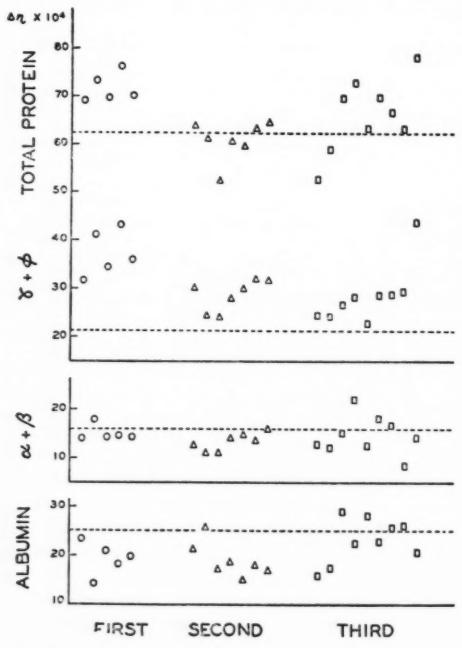

FIG. 5.—Plasma protein concentrations of chickens inoculated with inactive tumor tissue RPL 17.

First transfer, ○: inoculated on 35th day, bled on 117th day.

Second transfer, △: inoculated on 41st day (20 days after first transfer), bled on 114th day.

Third transfer, □: inoculated on 39th day (14 days after second transfer), bled on 114th day.

— — — — —: average values for 14 normal chickens 91 days old.

Inoculation with Inactive Tumor Tissue

A specimen of tumor tissue RPL 17 from the U. S. Regional Poultry Research Laboratory proved inactive. This strain was carried through three passages without demonstrating tumors macroscopically at necropsy 10 to 12 weeks after inoculation. Plasma obtained from the animals of the first passage showed abnormalities in protein composition similar to those found during tumor regression: reduced concentrations of albumin and increased concentrations of gamma globulin (Fig. 5). These deviations from the normal were less pronounced in the chickens of the second transfer, and still less in those of the third. The average albumin:globulin ratio rose from 0.378 for the first transfer to 0.479 for the second and 0.549 for the third.

Injection of Normal Liver Tissue

To test whether liver tissue contaminating the inactive tumor tissue used as inoculum might have contributed to the plasma changes, several chickens were injected with minced normal liver tissue. None of these chickens gave evidence for a drop in albumin concentration, and only one showed a significant increase of gamma globulin (Fig. 1, × □, 91 days).

Liver Damage

To determine whether the plasma changes accompanying tumor growth were the result of liver damage resulting from metastatic infiltration, 14 chickens were exposed to carbon tetrachloride vapors thrice daily for 12 days during a 2 week period. Two ounces of carbon tetrachloride were used for each animal, and the vapors were administered in a wooden box, about 4,000 cu. in. in capacity, equipped with a small motor-driven fan and a window for observation. Immediately at the onset of anaesthesia (usually after about ten minutes) the animal was removed. At necropsy the livers were enlarged and very pale, in color a light brown, and the kidneys appeared to be somewhat reduced in size.

In spite of the liver damage, none of the five

chickens surviving the treatment showed a decrease in albumin concentration (Fig. 6). The reduced albumin concentrations which accompanied tumor development, therefore, appear not to have been directly attributable to concomitant liver damage.

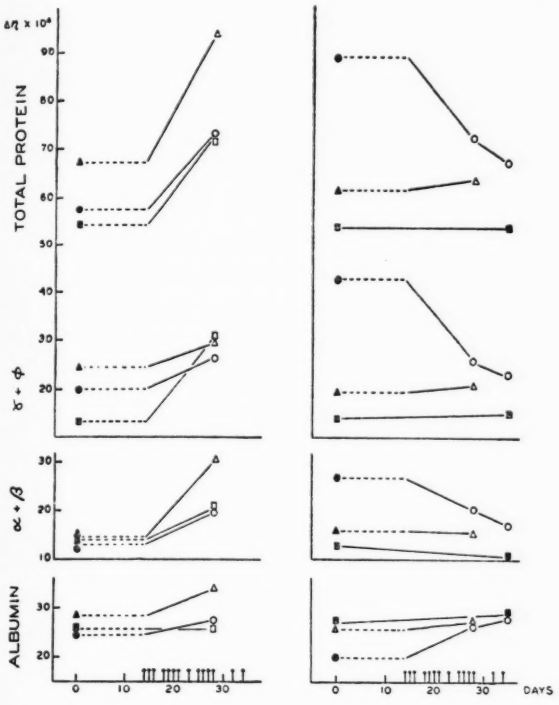

FIG. 6.—Effect of exposure to carbon tetrachloride vapors on the plasma protein concentrations. Points referring to the same chicken are connected by lines. Solid black symbols: untreated chickens. Line symbols: chickens exposed to carbon tetrachloride vapors at the times indicated by arrows.

X-IRRADIATION OF TUMOR-BEARING CHICKENS

X-irradiation of tumor-bearing chickens decreased the rate of growth of the tumor. The doses used were apparently of no therapeutic value, however, for the irradiated animals died earlier than the non-irradiated controls. Although too few electrophoretic experiments were carried out to warrant definite conclusions, the gamma globulin concentrations appeared to be increased, as in normal rats (13).

DISCUSSION

All but two of the 328 chickens inoculated with the RPL 16 strain of lymphoma developed tumors. Unless spontaneous regression occurred, the tumors proved fatal within 2 or 3 weeks.

The changes in plasma composition which accompanied the development and the regression of the lymphomas were consistent and reproducible. Rapid growth of the tumors was accompanied by a reduction in albumin concentration; in very young chickens, or in chickens which for some other reason were apparently more susceptible, there was a reduction of all the globulins as well. When tumor regression occurred, there was an abrupt rise in the concentration of gamma globulin, which appeared to be greater the longer regression was delayed, and which occasionally included the alpha and beta globulins as well. Complete regression appeared to be followed by a return to the normal. The plasma changes following inoculation of inactive tumor tissue were similar to those found during tumor regression.

The factors directly responsible for the albumin depletion accompanying rapid tumor growth are difficult to assess. While the reduction in albumin concentration might reasonably be attributed to the failure or impairment of albumin synthesis as a result of liver damage caused by massive tumor infiltration, it was not duplicated in chickens subjected to liver damage by exposure to carbon tetrachloride vapors. The severe depletion of albumin during tumor growth may be due to increased utilization of the albumin, or to competition for the materials from which it is synthesized, by the rapidly dividing tumor cells for their growth, and by the lymphocytes evoked by the host in response to the disease.

During regression the lymphoidal-like elements that compose the tumor undergo destruction. The lymphocytes and lymphocytic tissue elaborated by the host in response to the disease may also be destroyed. Recent evidence indicates that lymphocytes and lymphocytic tissue are a source of gamma globulin (3), which can be liberated into the plasma through adrenal cortical stimulation or by direct destruction of the tissue (13). Penetrating radiations are known to cause lymphocyte destruction (12, 7), and increased concentrations of gamma globulin have been observed in rats following large doses of x-rays (13) and in chickens following irradiation with neutrons (4). The large increase in gamma globulin which was found to accompany tumor regression may thus have been a direct result of the spontaneous destruction of the lymphoidal-like cells and lymphocytes produced during tumor growth.

The few chickens which exhibit regression and thus survive inoculation with the lymphoma appear to be immune to further implantation of the same strain (1). The extent to which immune bodies compose the gamma globulin produced during regression has not yet been determined. Sanders, Huddleson, and Schaible (8) attributed the elevated concentrations of gamma globulin in their leucosis-affected chickens to the formation of an antibody which they called the L component. Since in the

chicken, however, gamma globulin is the plasma constituent most susceptible to change, even in pathological conditions which do not elicit antibody formation (4), the increase during regression may consist to a large extent of non-specific protein.

SUMMARY

Electrophoretic analyses were made of the blood plasma of chickens inoculated with the RPL 16 strain of lymphoma. Rapid tumor growth was consistently accompanied by a reduction in albumin concentration, occasionally by a reduction in the concentrations of the globulins as well. The concentration of gamma globulin rose abruptly when tumor regression occurred; complete regression appeared to be followed by a return to the normal. The inoculation of inactive tumor tissue caused plasma changes similar to those found during tumor regression.

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Effect of Adrenal Cortical Extract on the Growth of Certain Mouse Tumors*†

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I. Transplanted Mouse Sarcoma 37

In the course of experiments designed to test whether adrenal cortical extracts would reduce the toxicity of *Serratia marcescens* polysaccharide (11) for Carworth Farms white mice bearing sarcoma 37 subcutaneously, it was noted that in one group of control mice injected intraperitoneally with 0.5 cc. of beef adrenal extract,[1] there was a noticeable decrease in size of the tumor and a high incidence of sloughing. Further experiments demonstrated that this effect was practically confined to female mice, and that the gross changes in the appearance of the tumor in female mice were accompanied by equally striking histological changes. While the total number of mice examined has been rather limited, the removal of one of the authors (L.V.B.) to another city made it seem desirable to publish the data obtained up to this time.

Since some of the tumors implanted into this strain of mice underwent spontaneous regression and sloughing, to the point of disappearance, the difference in incidence of disappearance of the implanted tumor has been used as one criterion of the effectiveness of the beef adrenal extract in producing damage to sarcoma 37 in Carworth Farms mice. Histological observations have furnished a second criterion (see below). The results on regressions are summarized in Tables I, III and IV. 0.5 cc. of extract was given intraperitoneally, usually in divided 0.1 or 0.25 cc. injections about one hour apart.

Decrease in size of tumor following injections of beef adrenal extract was noted in both male and female mice. However, the inhibition of tumor growth was temporary in the males, and could not be maintained even by repeated doses of extract, whereas, in our first experiment, in 17 of 19 females the tumor disappeared, and could not be detected at autopsy more than 4 months after the adrenal extract injections. Using Chi Square we have calculated that the probability of getting disappearance of the tumor in 17 of 19 female mice due entirely to spontaneous regression when 16 of 60 uninjected female mice showed similar disappearance of the tumor is less than 2 in 10,000. Dr. Katherine A. Brownell and her co-workers at Ohio State University have informed us that certain adrenal cortical fractions employed by them also inhibited the growth of sarcoma 37.

Table I: Effects of Adrenal Extracts on Growth of Sarcoma 37 in Carworth Farms Mice

Sex of mice	Treatment of mice	No. of mice so treated	No. showing tumor regression*
Female	One 0.5 cc. dose of beef adrenal extract† 7 to 10 days after tumor implantation	19	17
	None (controls for above)	60	16
Male	One 0.5 cc. dose of beef adrenal extract† 7 to 10 days after tumor implantation	29	5
	None (controls for above)	28	4
	Two 0.5 cc. doses of extract weekly until mouse died or tumor disappeared	10	1

* Tumor regression was judged to have occurred if the tumor had disappeared grossly and no tumor tissue could be detected on gross examination at autopsy. Control mice showing such regression were kept at least 5 weeks after tumor implantation before sacrifice. The 17 adrenal-extract-injected mice from which the tumor disappeared were kept for more than 4 months before sacrifice.
† Upjohn Pharmaceutical Company.

The spontaneous regressions of sarcoma 37 which we have observed did not occur during the first two weeks following tumor implantation. At 7 days after tumor implantation, caliper measurements gave identical average values for calculated tumor volumes in male and female mice. However, 2 and particularly 3 weeks after tumor implantation the tumors in the male mice were definitely larger than those in the females. Average length of life after implantation for mice dying with large tumors was about 3 weeks longer for females than for males.

* Aided by a grant from the Donner Foundation.

† A report of some of these results was presented at a meeting of the Philadelphia Physiological Society (1) in May 1946.

** Now at the National Cancer Institute, Bethesda, Md.

[1] We are indebted to Upjohn Pharmaceutical Company for beef adrenal extract.

TABLE III: GROWTH OF SARCOMA 37 IN CARWORTH FARMS FEMALE MICE FOLLOWING INTRAPERITONEAL INJECTIONS* OF UPJOHN CO. BEEF ADRENAL EXTRACT (GROUP I) OR 10% ALCOHOL (GROUP II)

	Period after IP injections							
	0–4 days		4–11 days		11–17 days		17–35 days	
	Group I	Group II	Group I	Group II	Group I	Group II	Group I	Group II
Tumor apparently gone	0/22	0/19	4/22	2/19	9/22	5/19	11/22	5/19
Tumor smaller	2/22	2/19	5/22	3/19	1/22	0/19	0/22	0/19
Tumor little changed in size	7/22	7/19	4/22	0/19	1/22	1/19	1/22	0/19
Tumor larger	13/22	10/19	8/22	14/19	8/22	11/19	1/22	3/19
Mouse dead	0/22	0/19	1/22	0/19	3/22	2/19	9/22	11/19

* 5 injections per mouse, 0.2cc per injection, 1.5 hours between successive injections.

Spontaneous regression of sarcoma 37 has not been observed in either male or female CAF_1 mice in the laboratories of the Chemotherapy Section, National Cancer Institute. The spontaneous regression observed by us indicates that sarcoma 37 was somewhat precariously established in the Carworth Farms strain, particularly in the females. This in turn suggests that the extensive tumor damage found in female mice following adrenal extract injections may have been due to an intensification of a definite tendency toward regression characteristic for this tumor, particularly in the females of the Carworth Farms strain, under the environmental conditions obtaining in the Philadelphia laboratory where the experiments were performed. We therefore repeated the experiments at the Bethesda laboratories, using Sarcoma 37 implanted in Carworth Farms Stock (Table 3) and in CFW_1 female mice (Table 4). Though the results were far less striking than those observed a year earlier, a similar type of result was obtained.

Histological observations on sarcoma 37.—Ten females bearing 7-day implants, 8 to 10 mm. in diameter, were injected intraperitoneally with 0.1 cc. of Upjohn beef adrenal extract hourly for 5 hours. In 5 mice sacrificed, 6 hours after the initial injection, the tumors appeared grossly hemorrhagic; whereas in 4 of 5 mice sacrificed after 25 hours, the tumors were not only hemorrhagic, but also shrunken and hard. In one mouse, only the encapsulating membrane, enclosing a collapsed bloody sac, remained at the tumor site.

Samples of tissue from at least 3 areas of the tumor were prepared for acetic orcein smears routinely. The necrotic residue of the original implant was discarded, and care was taken to select any clear and translucent tissue, since (in our experience) such tissue is the site of growing cells, and is usually free of spontaneous necrosis. If hemorrhage occurred in only part of the tumor, samples of hemorrhagic and nonhemorrhagic areas were prepared.

Smears of tissues dissected at 6 hours after the first hormone injection revealed fragmentary degeneration of nuclear substance, with pycnotic and occasionally vacuolated nuclei, and shrinkage of both nucleus and cell body in 3 of 5 tumors. (Fig. 1, *B*). The fourth tumor was apparently undamaged and undergoing cellular division; the fifth was partially affected, but contained an abundance of resting, apparently unharmed cells. At 25 hours after the first hormone injection, no mitosis was detected either in smears or in paraffin preparations from 4 out of 5 tumors. In a majority of these preparations the cells were broken down, and hazy, pycnotic nuclei were intermingled with degenerating nuclear fragments (Fig. 1, *D*). In some of the smears taken at 25 hours, some cells appeared

TABLE IV: ADRENAL EXTRACT EXPERIMENT OF 5/12/48

Tumors Implanted 5/6/48
Strain and sex of mice used: CFW_1 NIH ♀S

Survivals on	Adr. Extr. injected mice	10% alcohol injected mice	Non-injected mice	Remarks
5/24	10/10	10/10	4/5	
5/28	10/10	10/10	4/5	
6/2	10/10	8/10	3/5	
6/4	10/10	7/10	3/5	
6/5	10/10	6/10	3/5	All surviving mice appear to have shed all or by far the greater part of their tumors.
6/11	10/10	6/10	3/5	Two of mice in Adr. Extr. group show large tumors, also one of the mice in the non-injected group.
6/22	9/10	6/10	3/5	Three in Adr. Extr. group with large tumors. One in 10% alc. group with small tumor. One in non-inj. group with huge ulcerating tumor.
6/30	6/10	6/10	2/5	None in Adr. Extr. group with tumors. One in 10% alc. group with large tumor. Neither in non-inj. group have tumor.
6/30	Experiment terminated; mice sacrificed.			

intact but with nuclei condensed and the nuclear membrane crenelated.

In all of the smears the most striking difference from preparations of untreated tumors was that of staining. With acetic orcein, normal nuclei became sharp, clear magenta. In tumors from mice injected with adrenal cortical hormone, staining was indefinite, unclear, and the structures were hazy and uncertain of outline (Fig. 2, *A*). Intermingled with the opaque, hazily stained cells, were formless chromatin fragments (Figs. 1, *D* and 2, *B*).

In another experiment performed many months later, 15 female mice bearing 12 to 15 mm. tumors (sarcoma 37) were treated on 2 successive days with a total of 3 doses (0.75 cc.) of Upjohn beef adrenal extract; one week later the tumors were dissected for microscopic study in orcein smears and in paraffin sections. In the interim two animals died; of the remaining 13 animals, 1 had already completely sloughed its tumor. In 5 mice the tumors, which were only slightly diminished in size, as judged by external measurements, were found to comprise nothing but dead tissue, white and opaque, and puckered in folds which were adherent to the inner surface of a scab. No normal-appearing cells were observed in slides made from these 5 tumors. In 3 mice the tumors, already partially sloughed, were filled with degenerating cells, and no division could be detected. Some seemingly normal cells remained in 3 mice although the bulk of the tissue was moribund. In only one of the 13 surviving mice was the tumor growing rapidly with no signs of hemorrhage or sloughing. The tumors in the 15 controls meanwhile continued to grow; 3 had broken through the skin and 1 of these 3 was partially cavitated. Paraffin sections of the cavitated tumor showed central and focal necrosis. Also revealed were extensive areas of dividing tissue, not only at the periphery but in growth centers distributed throughout the tumor.

II. Experiments Using Methylcholanthrene Induced Inflammatory Sacs and Sarcomas

Sarcomas were induced on the dorsal surface of A strain female mice by subcutaneous injection of 0.25 mgm. of methylcholanthrene dissolved in 0.1 cc. of lard filtrate, prepared as described by Leiter and Shear (8). Within 4 to 6 weeks, inflammatory sacs developed at the site of the methylcholanthrene injection. Mice were sacrificed from time to time to permit study of the inflammatory sacs. These studies revealed that the interior of these sacs may be filled with fluid, with non-cellular solid material, or both, and that strands of tumor tissue gradually envelop, penetrate into, and replace the materials present in the interior of the inflammatory sacs. Histological descriptions of the change from inflammatory sac to tumor are being published separately. In our experience some inflammatory sacs containing little tumor tissue felt as solid on palpation as other masses composed almost entirely of tumor tissue. Our observations indicate that a methylcholanthrene-induced mass with an estimated volume of 0.1 cc. generally contains appreciable amounts of both tumor tissue and inflammatory sac material, and that a mass of estimated volume more than 0.3 cc. is generally composed mainly of tumor tissue. Attainment of a volume of 0.3 cc. for the methylcholanthrene-induced mass required 13 to 21 weeks. Estimations of these volumes were made by use of the formula for a half spheroid ($V = 0.524\ lwh$, where l, w and h = caliper measurements, in cm., of the length, width and height of the mass).

After the masses exceeded 0.1 cc. they were matched for size. One mouse of each pair was given intraperitoneal injections of Upjohn beef adrenal extract, 0.1 cc. daily, 6 days a week, and the control mouse was given similar injections of 10% alcohol (in the Upjohn beef adrenal extract the steroids are dissolved in 10% alcohol). The injections were continued until the mouse died or (in 1 case) the methylcholanthrene-induced mass disappeared.

The data obtained are summarized in Table II. Original volumes of methylcholanthrene-induced tumors refer to those calculated at the time that injections were begun. K values for the rates of increase (or decrease) in size of the methylcholanthrene-induced masses were calculated, using the equation $K = \frac{\ln V_2 - \ln V_1}{(t_2 - t_1)}$. Each average value in the Table is followed by its standard error. No claim is made here of accuracy for either the volumes of the induced masses or their K values. The K values are employed in the Table to show that when the adrenal extract was administered in the manner described above to mice bearing definitely established and rapidly growing sarcomas, *i.e.*, methylcholanthrene-induced masses of volumes greater than 0.3 cc., the extract had no demonstrable effect on the rate of growth of the tumors.

The data for the methylcholanthrene tumors of an original volume less than 0.3 cc. might be interpreted as indicating an inhibitory effect of the

TABLE II: RATES OF INCREASE IN SIZE OF METHYLCHOLANTHRENE-INDUCED TUMORS AND LONGEVITY OF MICE BEARING THESE TUMORS IN RELATION TO INTRAPERITONEAL INJECTIONS OF (A) ADRENAL CORTICAL EXTRACT OR (B) 10 PER CENT ALCOHOL (CONTROL MICE)

Group no.	Orig. volumes of induced tumors, cc.	Treatment	No. of mice in group	No. with 20 day decr. in size of mass	Avg. *K* value for group (1st 20 days)	Avg. longevity, days	
						After methylchol. inj.	After begin. of extr. or alc. inj.
I	0.09–0.25	Adr. extr. inj.	8	3*	0.021 ± 0.024	168.4 ± 7.6†	55.3 ± 7.4†
II	0.09–0.24	10% alc. inj.	9	1	0.098 ± 0.013	149.4 ± 8.6	40.2 ± 7.1
III	0.51–2.2	Adr. extr. inj.	9	0	0.121 ± 0.015	129.0 ± 4.0	24.0 ± 3.1
IV	0.32–3.6	10% alc. inj.	9	0	0.102 ± 0.012	130.8 ± 8.3	23.4 ± 3.8

* Tumor disappeared in 1 instance.
† Data for mouse from which tumor disappeared were excluded.

adrenal extract injections on the rate of growth of very small tumors. However, because of the presence of considerable and varying amounts of inflammatory sac material in these small methylcholanthrene-induced masses, it seems inadvisable to us to use them in an evaluation of the effects of agents on tumor growth.

When life durations from the beginning of the adrenal extract or alcohol injections were plotted against volumes of the methylcholanthrene-induced masses at the time when these injections were begun, a hyperbolic curve was obtained, demonstrating for both experimental and control mice a negative correlation between life expectancy and the amount of tumor tissue present. This effect was so striking that we believe reliable data on agents affecting the longevity of mice bearing methylcholanthrene-induced sarcomas can be obtained only if care is taken to select large numbers of mice bearing tumors of practically identical size for the experimental and control groups. The slightly greater average life span for mice in Group I (Table II) as compared with those in Group II can, therefore, be interpreted only as suggestive of an effect which might be demonstrable by employing large numbers of mice bearing very small methylcholanthrene-induced masses that contain questionable amounts of tumor tissue.

Dobrovolskaïa-Zavadskaïa and Zephiroff (2) state that adrenal extracts prepared by them induced a decrease in the rate of growth of both spontaneous mouse adenocarcinomas and benzpyrene-induced mouse sarcomas, and an increase in longevity of the hosts. They give no average values, and no data for tumor sizes in either the control or the experimental series at the time when the adrenal extract injections were begun. Their published data are, therefore, inadequate for the reader to draw any independent conclusions regarding a possible effect of their adrenal extracts on either the rate of tumor growth or longevity of the mice bearing these tumors.

III. HISTOLOGICAL OBSERVATIONS ON METHYLCHOLANTHRENE INDUCED PRIMARY TUMORS IN MICE INJECTED WITH LARGE AMOUNTS OF BEEF ADRENAL EXTRACT

Microscopic observations by the methods already described for sarcoma 37 gave definite indication of abnormalities in the tumor tissue of methylcholanthrene-induced tumors in mice injected with large doses of Upjohn beef adrenal extract.

Beginning 170 days after the methylcholanthrene injection, each of 5 mice was injected daily with 0.5 cc. of the extract for 7 days (total, 3.5 cc.). The excised tumors showed a greatly altered growth pattern. Instead of the long, continuous chains of spindle-shaped cells (Fig. 2, *C*) the tumors from these treated mice contained cells which were foreshortened, generally aberrant and arranged in a confused and chaotic fashion (Fig. 2, *D*). Some nuclei were fragmented or pulled out into thread-like processes uncommon in these tumors. In 1 tumor which appeared totally coagulated, the nuclei were very darkly stained and pycnotic; in others no dividing cells could be demonstrated by our technics. Even in those masses where dividing cells were present, the metaphases were clumped and abnormal. In only 1 tumor of this series were the cells actively dividing.

Six mice bearing practically stationary tumors were injected with 0.5 cc. of Upjohn extract on the first day and thereafter with 0.25 cc. every other day for 2 weeks (total 2.50 cc.) Injections were started approximately 3 months after the application of the carcinogen and in all but 1 tumor some residual sac material was found at the close of the experiment. Again, the growth pattern was interrupted and chaotic. Degeneration of cells was notable, particularly as regards the cytoplasm, which was ragged and vacuolated. Where no dividing cells were observed, the resting nuclei were shrunken and scattered through a discontinuous and formless cytoplasm. One mass (inflammatory

sac plus tumor tissue) shrank to a point where it was no longer measurable through the skin. The minute fragment dissected for microscopic study was filled with degenerating cells.

DISCUSSION

Papers by Murphy and Sturm (9, 10), by Heilman and Kendall (6), and the results reported here, demonstrate that certain transplanted animal tumors undergo marked regression following administration of adrenal cortical steroids. For 2 of these tumors it was noted that the regressing action of the steroids was much more pronounced in female than in male mice (the lympho-sarcoma of Heilman and Kendall and sarcoma 37). However, from the reports of Woglom (13) and Sugiura (12), among others, and the unpublished findings of still other research workers (5), it would appear that adrenal cortical extracts and pure steroids, when given in any amounts practicable at the present time, would have relatively little effect on the growth of the great majority of transplanted tumors.

The only satisfactory evidence indicative of a definite (temporary) regressing action of adrenal cortical steroids on a nontransplanted type of tumor, of which we are aware, is that presented in a recent paper by Law and Speirs (7) describing certain effects of lipoadrenal extract (Upjohn) on spontaneous lymphoid leukemias in mice. The known effects of adrenal cortical steroids and the adrenocorticotropic hormone in producing lymphocytopenia and involution of normal lymph nodes (3, 4) led Law and Speirs to their study of the effects of adrenal extracts on mouse lymphatic leukemias.

SUMMARY

1. Mouse sarcoma 37, implanted subcutaneously into Carworth Farms mice, showed some spontaneous regression and sloughing. Intraperitoneal injection of adrenal cortical extract greatly increased this reaction in female but not in male mice.

2. Repeated intraperitoneal injections of this same extract had no demonstrable effect on the growth of definitely established methylcholanthrene-induced sarcomas.

3. The methylcholanthrene-induced sarcomas did show histological abnormalities in mice receiving large repeated injections of the beef adrenal extract.

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DESCRIPTION OF FIGURE 1

FIG. 1.—*A*—Acetic-orcein smear of mouse sarcoma 37, untreated. Mag. × 900. *B*—Sarcoma 37 from female mouse injected with beef adrenal cortical extract, 6 hours after initial injection. Mag. × 900. Nuclei have become vacuolated and pycnotic. *C*—as for *B;* note shrinkage and vacuolization of nuclei and haziness of staining; division still in progress. Mag. × 900. *D*—Sarcoma 37 from female mouse injected with beef adrenal cortical extract. Smear taken 25 hours after initial injection. Mag. × 900. Hazy, pycnotic nuclei are intermingled with degenerating nuclear fragments.

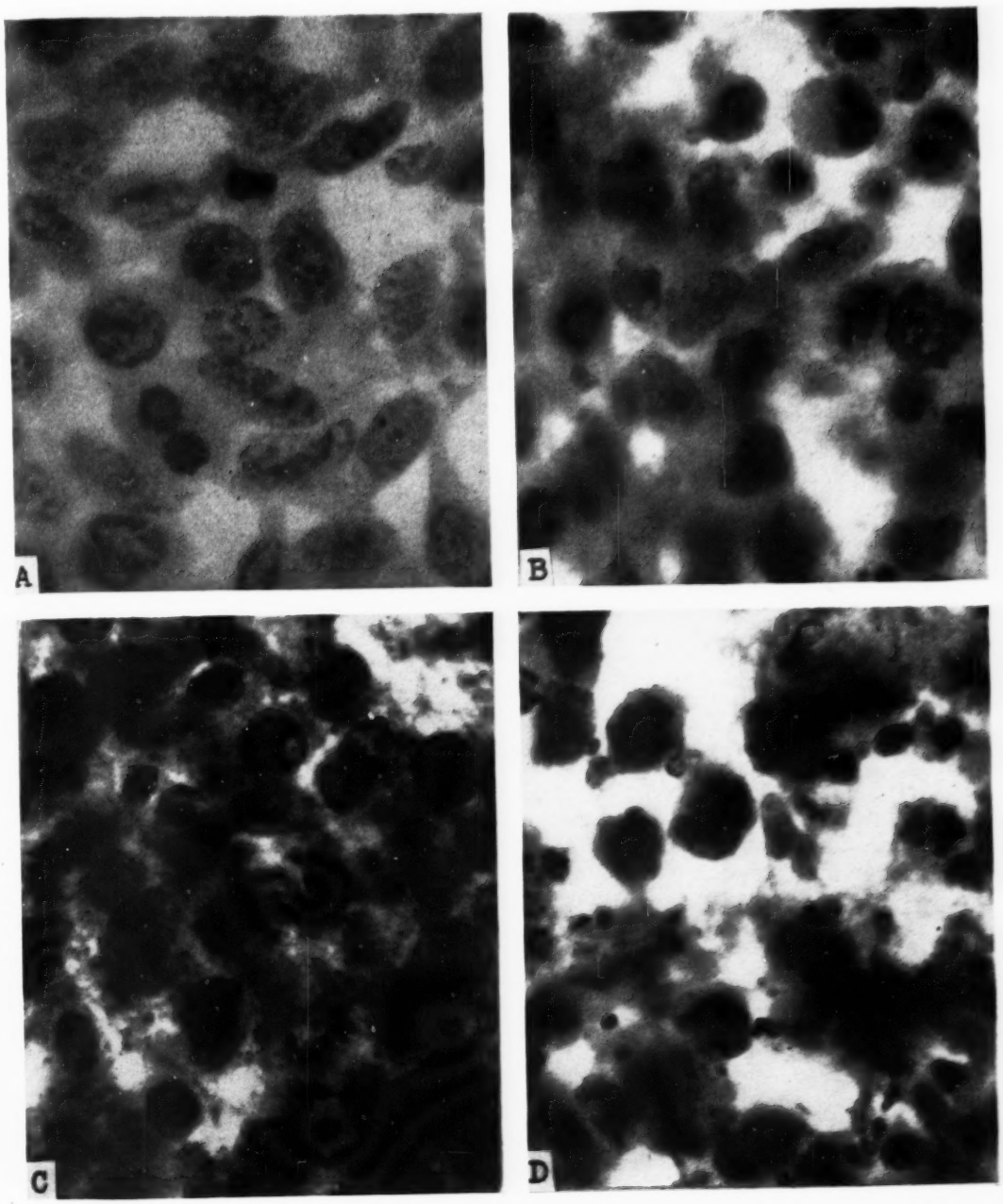

FIG. 1

DESCRIPTION OF FIGURE 2

FIG. 2.—*A*—Acetic-orcein smear of mouse sarcoma 37. Mag. × 600. A hemorrhagic tumor, 25½ hours after injection with 0.5 cc. of beef adrenal extract. *B*—the same, 72 hours after initial injection. Mag. × 180. Note nuclear ghosts and granular debris. *C*—Paraffin section (material fixed in Allen's fluid and stained in hemalum and eosin) of untreated primary mouse tumor induced by subcutaneous injection of methylcholanthrene in lard filtrate, 98 days after application of the carcinogen. Mag. × 600. *D*—Same material as in *C*, treated with beef adrenal extract, 0.1 cc. daily for 1 week (total 0.8 cc.) Mag. × 600. Growth pattern is disrupted and tissue generally degenerating.

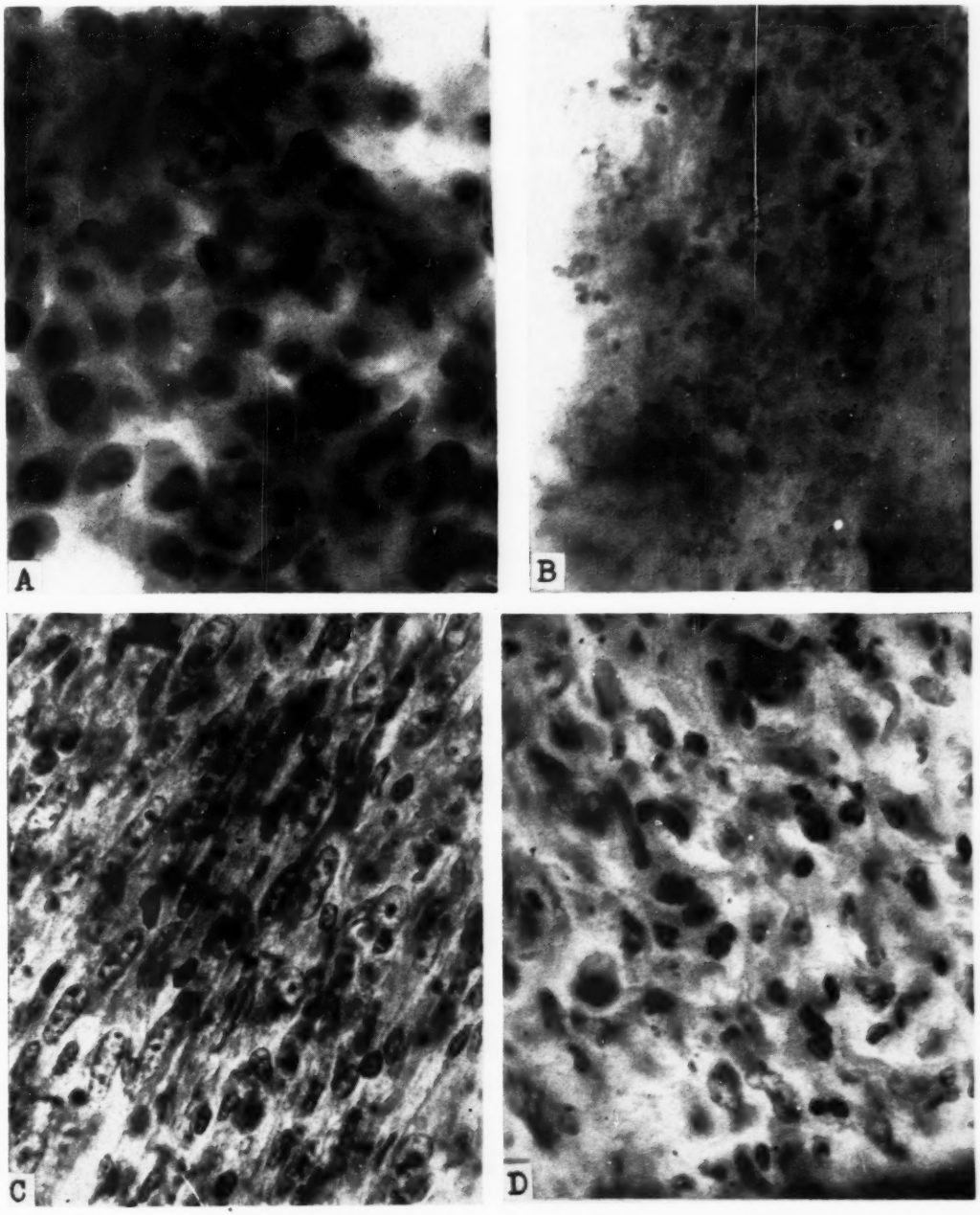

FIG. 2

Histological Changes in Adrenal Glands of Tumor-Bearing Mice Injected with *Serratia marcescens* Polysaccharide Alone and in Combination with Adrenal Cortical Extract

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As has been reported previously by Diller and Shear (6) and by Diller (4), tumor cells of mice injected with a polysaccharide derived by Shear (9) from *Serratia marcescens* culture filtrate show degenerative responses within 6 hours. At a dose level that produced rapid sloughing of sarcoma 37 in about 1 out of every 4 mice, an equal number of mice usually succumbed to treatment. When adrenal cortical extract was administered simultaneously, the toxic effect of the polysaccharide was measureably reduced (2). This did not interfere with tumor sloughing. Adrenal extracts alone are also capable of producing degenerative changes in sarcoma 37 and sloughing of the tumor, particularly in female mice (1, 5).

Injection of *S. marcescens* polysaccharide in tumor-necrotizing dosage into mice bearing sarcoma 37 was followed in 5 or 6 hours by hemorrhage, not only in the tumors but also in the adrenal glands. The adrenal hemorrhage was accompanied in these glands by destructive cellular changes which were most severe in the medullary region. Milder histological changes occurred in the adrenal glands of mice given polysaccharide plus adrenal cortical extract. The changes are described below.

MATERIALS AND METHODS

We selected as tumor hosts, young albino mice, 3 to 4 months of age, both males and females, reared in our laboratory from Carworth Farms stocks. Most of the studies were made on animals implanted with sarcoma 37, but some experiments were performed also on mice with methylcholanthrene-induced tumors and on C3H mice bearing spontaneous mammary tumors. In the experiments with sarcoma 37, mice bearing 7 day implants were divided into 3 groups of 20 animals each, one of which received polysaccharide (Sample P-3); the second, Upjohn adrenal cortical extract; and the third, the two substances in combination. The amount of polysaccharide administered (0.02 mgm. intraperitoneally) was sufficient to cause extensive destruction of tumor cells in 3 to 5 tumors of every 10, while causing the death of 2 or 3 of the mice within the first 24 hours. Mice of the group to be treated by adrenal hormone were given 0.5 cc. of Upjohn aqueous extract in 5 intraperitoneal doses of 0.1 cc. each 1 hour apart. The first dose was administered simultaneously with the polysaccharide injection. The control group received cortical extract only (0.5 cc. in 5 doses at hourly intervals). This experiment was later repeated with 15 animals in each of the 3 groups, using paired mice and female litter mates 3½ months of age. All of these animals were killed for histological study.

At the end of 6 hours, at which time polysaccharide alone produced gross hemorrhage in the tumor, 5 animals from each group were killed and the tumors as well as the adrenal glands were dissected for microscopic study. At 24 to 25 hours after initial treatment 5 more animals from each group were similarly sacrificed. The remainder of the animals were allowed to live for survival studies and tumor measurement (5).

The tumors were prepared for microscopic study by means of the acetic orcein technic; the adrenal glands by routine paraffin technics, stained with hemalum-eosin.

OBSERVATIONS

Six hours after injection of 0.02 mgm. of polysaccharide the adrenal glands of both male and female tumor-bearing mice showed definite cell changes which were most marked in the medullary region. Nuclei were greatly shrunken and the nuclear membranes crenulated (Figs. 1 and 2). The medullary tissue was disrupted and large open spaces sometimes appeared between the cells. There was infiltration of white blood cells in both cortex and medulla, particularly in the border region between the two primary zones, as well as along the radial septa of the cortex. Marked hemorrhage occurred

in some instances in the medulla. The extra-capsular connective tissue area showed destruction or degeneration, with some hemorrhage; the zona glomerulosa decreased in size and there was pycnosis and crenation of nuclei; the zona fasciculata increased peripherally at the expense of the glomerulosa; and the zona reticularis showed a tremendous increase in number of red blood cells at the line of demarcation between the cortex and the medulla. Nevertheless, there was less nuclear change in the cortex than in the medulla, where nuclear pycnosis and distortion, as well as cytoplasmic shrinkage and vacuolization, were common and accompanied by vascular degeneration. In some cases the entire tissue exhibited an unusual affinity for eosin.

Twenty-four hours after initial injection of 0.02 mgm. of polysaccharide, nuclear changes were not much greater than those noted at 6 hours, though the medulla usually exhibited great loss of nuclear chromatin, and cytoplasmic detail was indefinite because of cloudy staining. Refractile crystalline bodies were often present.

When the amount of polysaccharide was increased to 0.1 mgm. which produced a higher percentage of deaths, the adrenal glands from animals with markedly hemorrhagic tumors usually showed still greater histological changes, as illustrated in Fig. 2, A to D. Fig. 2, C and D show the cortex and medulla, respectively, from the left adrenal gland of a moribund animal 25 hours after intraperitoneal injection of 0.1 mgm. of polysaccharide. Destruction of the medullary region (Fig. 2 D) is here practically complete, the nuclear substance degenerating and the entire medullary area karyorrhexic. The cortex also is damaged, as shown in Fig. 2, C.

Unexpectedly, striking changes were found also in the adrenal glands of the control group of tumor-bearing mice injected with Upjohn extract only, though they were not as extensive in most cases as those arising from polysaccharide damage. An example of the extreme effects obtained appears in Fig. 3, C and D, which is from an adrenal gland of a 3 month old female mouse injected with a total of 0.5 cc. of Upjohn adrenal cortical extract over a 5 hour period. Here there is extensive hemorrhage in both medulla and cortex. The nuclei of the medulla were cloudy and reddish while those of the cortex were shrunken, distorted and apparently degenerated. The cytoplasm of the medullary cells was foamy and full of vacuoles. Connective tissue septa had broken down completely in the cortical region and nuclei were pycnotic and shrunken. The adrenal glands of female mice injected with cortical extract showed greater damage than those of males in most instances, as will be discussed later.

When polysaccharide and adrenal cortical extract were injected simultaneously, a balancing effect was apparently obtained. (See Fig. 4, A and B). Although there were some changes in both medulla and cortex, they were not as extensive as those which were produced by either substance alone. In the medulla the changes encountered were swelling of the cytoplasm, separation of cells, and nuclear distortion. Six hours after combined treatment, the zona glomerulosa and zona fasciculata were relatively normal but contained many polymorphonuclear leukocytes, while the zona reticularis was sometimes severely damaged and heavily infiltrated by white blood cells. Occasionally the latter zone was found to be greatly enlarged by crowding of red blood cells with which the entire region was usually engorged.

Twenty-five hours after simultaneous treatment with cortical extract and polysaccharide, degenerative changes in the adrenal glands had disappeared (Fig. 4, C and D). Whereas the capsule still showed vascular degenerative changes, the three zones of the cortex were practically normal (Fig. 5, D). The medulla here shows residual hemorrhage; however, the nuclear volumes appear to be not only reconstituted but even augmented, as if by uptake of fluid. Histological preparations stain sharply with nuclear dyes and the chromatin is concentrated within the medullary nuclei in definite, deeply stained granules.

Therefore the histological pattern of adrenal gland damage agrees rather closely with the physiological results obtained (2). Though polysaccharide in tumor-necrotizing doses may cause notable destruction of adrenal tissue, toxic effects are somewhat lessened by simultaneous administration of adrenal cortical extract.

In an attempt to determine whether or not adrenal response to these toxic substances was part of a general alarm reaction in which the pituitary gland might conceivably participate, histological studies were made also of other endocrine glands, including the pituitary, the thymus and the thyroid. No distinct response could be detected in any of these glands.

In the case of the thymus and the thyroid, hemorrhage occurred in the sustentacular connective tissue supporting the glands following polysaccharide injection, but no effect whatever could be observed in either tumorous or non-tumorous mice receiving 0.5 cc. of adrenal extract only. Following polysaccharide

injection (0.02 mgm.) the pituitary showed alterations in staining of the chromophile cells. The eosinophilic ceils became stained a vivid pink, while their granular character was lost in what appeared to be complete lysis of the cytoplasm. No changes were observed in these cells following injection of adrenal extract.

No mention has been made above of the effects of these substances on adrenal glands of mice which were not tumor-bearing. In some cases no damage at all could be detected in non-tumor-bearing mice, but where found the changes were, in every instance, less extensive. Compare, for instance, the difference between adrenal glands of a non-tumor-bearing mouse injected with 0.02 mgm. of polysaccharide (Fig. 1, A and B) with that produced in the glands of a tumor-bearing mouse of the same age and sex, injected with the same amount of polysaccharide, each excised at 6 hours after injection (Fig. 1, C and D). Even more striking is the difference between tumor-bearing and non-tumor-bearing mice injected with cortical extract alone (Fig. 3). In the case of the non-tumorous mouse there is pycnosis of the cortical cells and hemorrhage in the medulla; in the tumor-bearing mouse subjected to the same amount of cortical hormone for the same number of hours, extensive histological changes have taken place in both medulla and cortex. As noted above, these differences are particularly striking in female mice injected with cortical hormone, which agrees with our finding that a much higher degree of tumor destruction is attained in females than in males bearing sarcoma 37 following the injection of adrenal extract (5).

Further evidence in support of this interpretation is furnished by the responses of adrenal glands of C3H mice bearing spontaneous mammary carcinoma. Fig. 6 shows examples from a small series of such mice (8 in each group) injected with polysaccharide and with adrenal extract. This particular tumor does not respond with any degree of cellular destruction to either polysaccharide or cortical hormone treatment and the adrenal damage also appears to be negligible.

In Fig. 6, A and B, are the cortex and medulla of an untreated female bearing multiple mammary tumors of different sizes. C and D shows the cortex and medulla of another female mouse with one well developed mammary tumor, injected with 0.02 mgm. of polysaccharide. Shrinkage of cortical nuclei as well as of whole cells has occurred, and cell boundaries, so definite in the adrenal cortex of the healthy C3H mouse, are almost obliterated. The medulla also shows nuclear shrinkage and crenation as well as hemorrhage. These changes are much less severe than those appearing in albino mice with transplanted tumors, subjected to the same amount of polysaccharide (Fig. 1, A and B) but tumor-necrotization in the case of these primary carcinomas is also very slight, which lends further support to our assumption that polysaccharide alone is not responsible for all the adrenal damage, but that the reaction is greatly enhanced by tumor breakdown. E and F illustrate the cortex and medulla from a female C3H mouse with one large mammary tumor treated with cortical extract only, and in which there was little tumor cell destruction.

DISCUSSION

The changes described in the foregoing pages, at least as far as polysaccharide is concerned, may of course be merely one manifestation of the alarm reaction described by Selye (8). In fact, he has listed bacterial toxins as one class of substances which can elicit such responses. Olitski, Avinery, and Koch (7) conducted a series of experiments with guinea pigs injected with toxins from many different bacterial groups and found that 24 to 48 hours after injection, or when animals died as a consequence of injection, the adrenal glands became grossly enlarged and hemorrhagic. Microscopically, the deeper layers of the cortex were hyperemic. Such reactions were produced only by certain groups of microorganisms. *S. dysenteriæ* and some other gram-negative bacteria produced in the guinea pig, within 24 hours post-injection, greatly enlarged adrenal glands, which were of a very dark red color. Usually these phenomena were accompanied by hypothermy, hemorrhage and leukopenia.

This agrees very well with what we have observed in tumor-bearing mice. *Severe* histological damage in the adrenals was encountered by us only in animals in which there was tumor necrosis and tissue breakdown.

When adrenal cortical extract is administered simultaneously with the polysaccharide, the adrenal picture, as previously noted, is much more nearly normal, despite the fact that tumors degenerate to about the same extent under the combined treatment. However, tumor cell destruction proceeds much more slowly, the maximum effect not being reached for 24 hours, instead of at 6 hours as with polysaccharide alone.

Apropos of the protective action of the adrenal extract, is Selye's observation(8, page 181) "that in animals overdosed with corticoids, the response

of the adrenal cortex to alarming stimuli is abnormal."

No explanation is proffered as to the uniformly more drastic response of the medulla than of the cortex, especially when adrenal cortical extract alone is injected. Why should even an overdose of adrenal corticoids cause degeneration of the medulla as severe as, for instance, that shown in Fig. 3, D?

According to Selye the chromaffin system "has been shown to play an important rôle only during the first few minutes of the alarm reaction—the discharge of the chromaffin granules which occurs during the shock phase may be secondary to dehydration."

The photographs in Fig. 3 indicate that in the non-tumor-bearing mouse 0.5 cc. of adrenal cortical extract is *not* an effective agent in eliciting the histological changes in the adrenals which are characteristic of the alarm reaction (Fig. 3, A and B, in which the histological appearance is normal). On the other hand, histological changes characteristic of the alarm reaction do occur in the adrenal glands following the injection of this amount of extract into mice bearing sarcoma 37 (Fig. 3, C and D). Since this particular tumor shows marked cellular degeneration following administration of this amount (0.5 cc.) of adrenal extract, it would appear likely that the changes in histological appearance occurring in the adrenals of sarcoma 37 mice following administration of adrenal extract are due to the presence in these mice of tumor tissue disintegrating under the action of the extract rather than (a) to a direct action of the extract on the adrenals of these mice, or (b) to a more generalized change in susceptibility of tumor-bearing mice. Further evidence for this point of view is provided by the fact that female mice bearing sarcoma 37 show more pronounced histological damage in both tumor and adrenals, following administration of adrenal extract, than do male mice bearing sarcoma 37.

SUMMARY

1. The intraperitoneal injection of *Serratia marcescens* polysaccharide causes enlargement of and hemorrhage in the adrenal glands of tumor-bearing mice. Histological changes of a degenerative nature also occur and these increase in severity in proportion to the dose.

2. Changes of the same nature are produced in the adrenal glands of host mice when adrenal cortical extract is used to produce necrotization of transplanted tumors.

3. When adrenal cortical extract is employed in combination with polysaccharide, tumor breakdown is delayed and the effect on the adrenal glands is minimized. At 24 hours after simultaneous injection of the two substances, both cortex and medulla appear relatively normal, except for residual hemorrhage.

4. In non-tumor-bearing animals, or in animals with primary tumors which do not respond to these agents through tumor degeneration, adrenal damage following polysaccharide, adrenal cortical extract, or the two in combination, is negligible.

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DESCRIPTION OF FIGURE 1

Fig. 1.—A—Cortex of adrenal gland of non-tumorous albino mouse (male), injected with 0.02 mgm. of *S. marcescens* polysaccharide. B—medulla of same mouse. C—Cortex from adrenal gland of male albino mouse bearing a 7-day sarcoma 37, 6 hours after injection with 0.02 mgm. of polysaccharide; D—medulla of same. Mag. × 440.

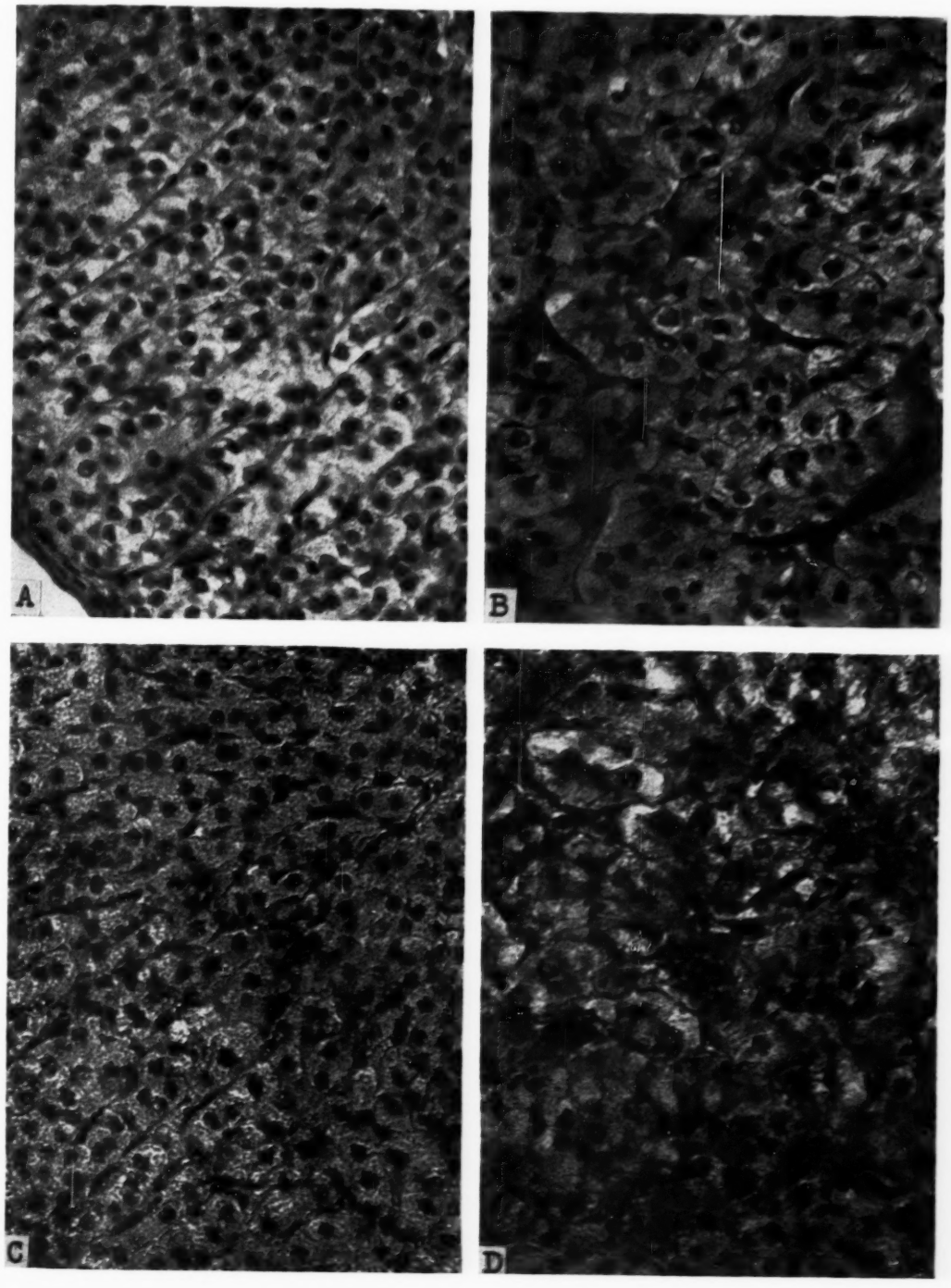

FIG. 1

DESCRIPTION OF FIGURE 2

FIG. 2.—Sections of adrenal glands from tumor-bearing male albino mice injected with 0.1 mgm. of *S. marcescens* polysaccharide. A and B—cortex and medulla, respectively, 6 hours after injection; C and D—cortex and medulla, respectively, 24 hours after injection. Mag. × 440.

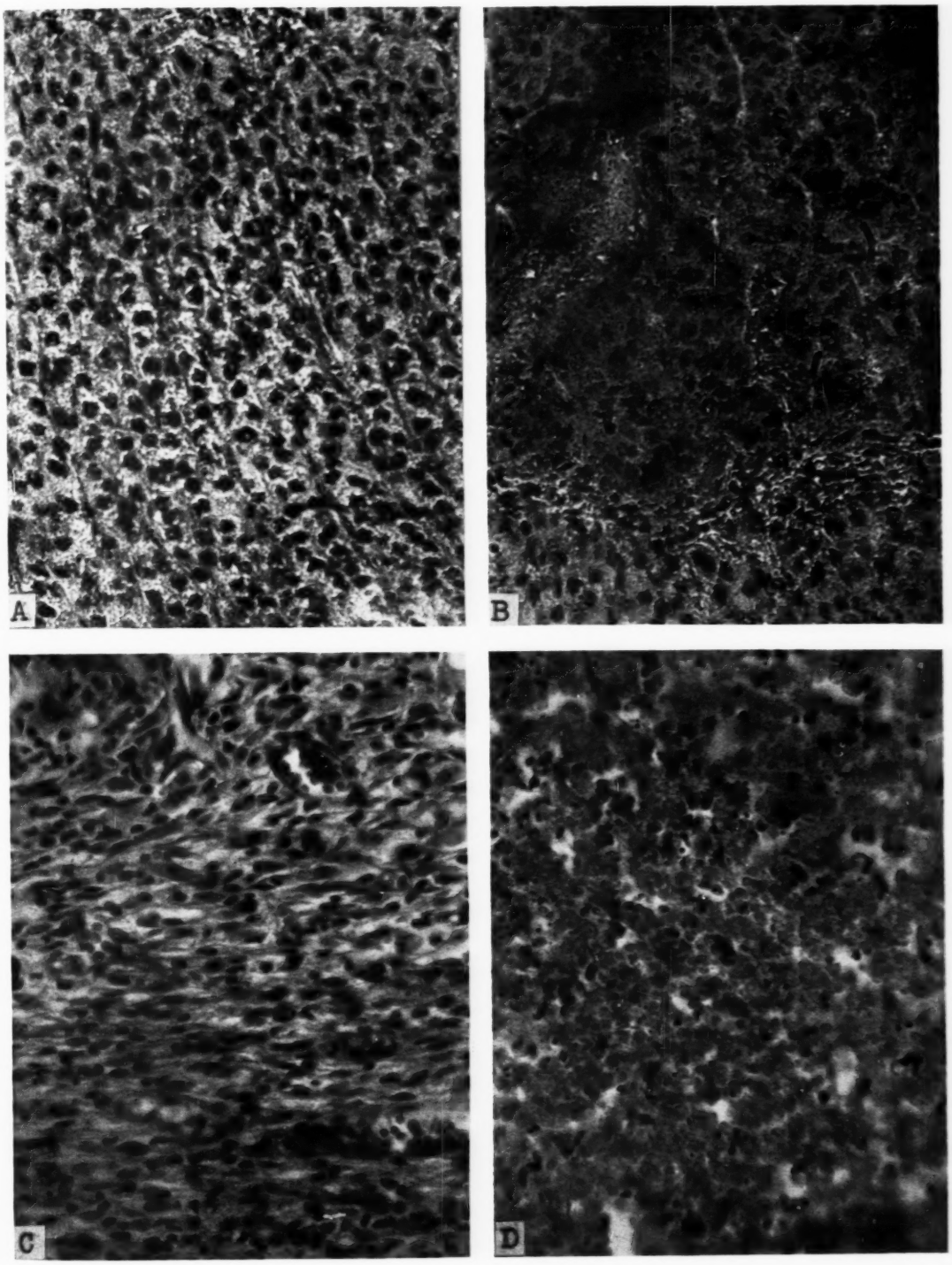

FIG. 2

DESCRIPTION OF FIGURE 3

FIG. 3.—Sections of adrenal glands from mice injected with Upjohn adrenal extract. A and B—cortex and medulla, respectively, of a nontumorous mouse. C and D—cortex and medulla of a female tumor-bearing mouse, injected in each case with 5 doses of 0.1 cc. each of Upjohn beef adrenal extract, 6 hours after initial injections. Mag. × 440.

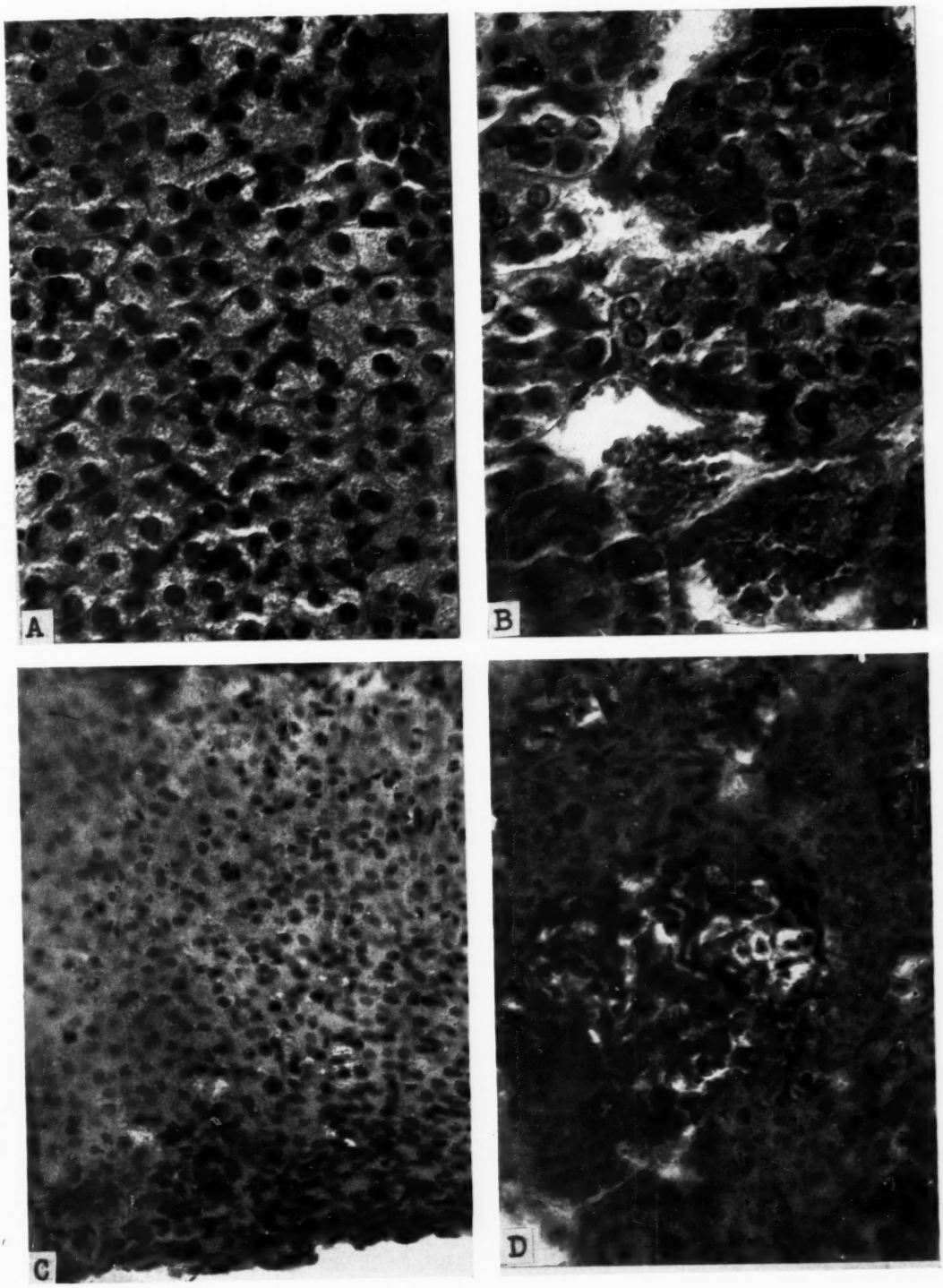

FIG. 3

DESCRIPTION OF FIGURE 4

FIG. 4.—Sections of adrenal glands from albino female mice implanted with sarcoma 37. A and B—cortex and medulla, respectively, of mouse treated simultaneously with *S. marcescens* polysaccharide and Upjohn beef adrenal extract, 6 hours after initial injection; C and D—cortex and medulla from mouse similarly injected, 24 hours after initial injection. Mag. × 440.

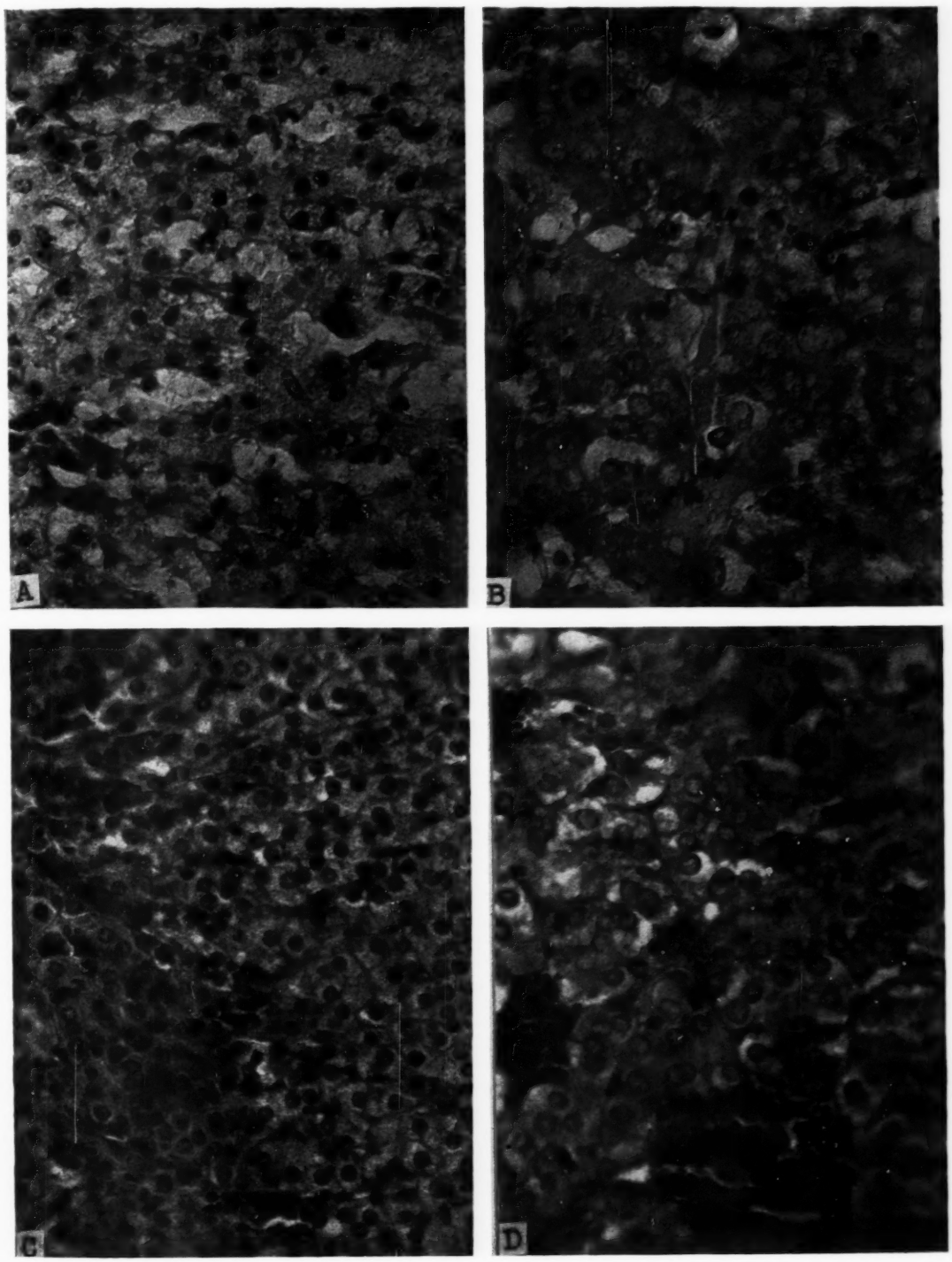

FIG. 4

DESCRIPTION OF FIGURE 5

FIG. 5.—Sections of adrenal glands, showing response of entire adrenal to polysaccharide, adrenal cortical extract, and the two in combination. A—untreated control. B—6 hours after injection with 0.02 mgm. of polysaccharide. C—6 hours after injection with 0.5 cc. adrenal cortical extract. D—25 hours after combined treatment. Mag. × 80.

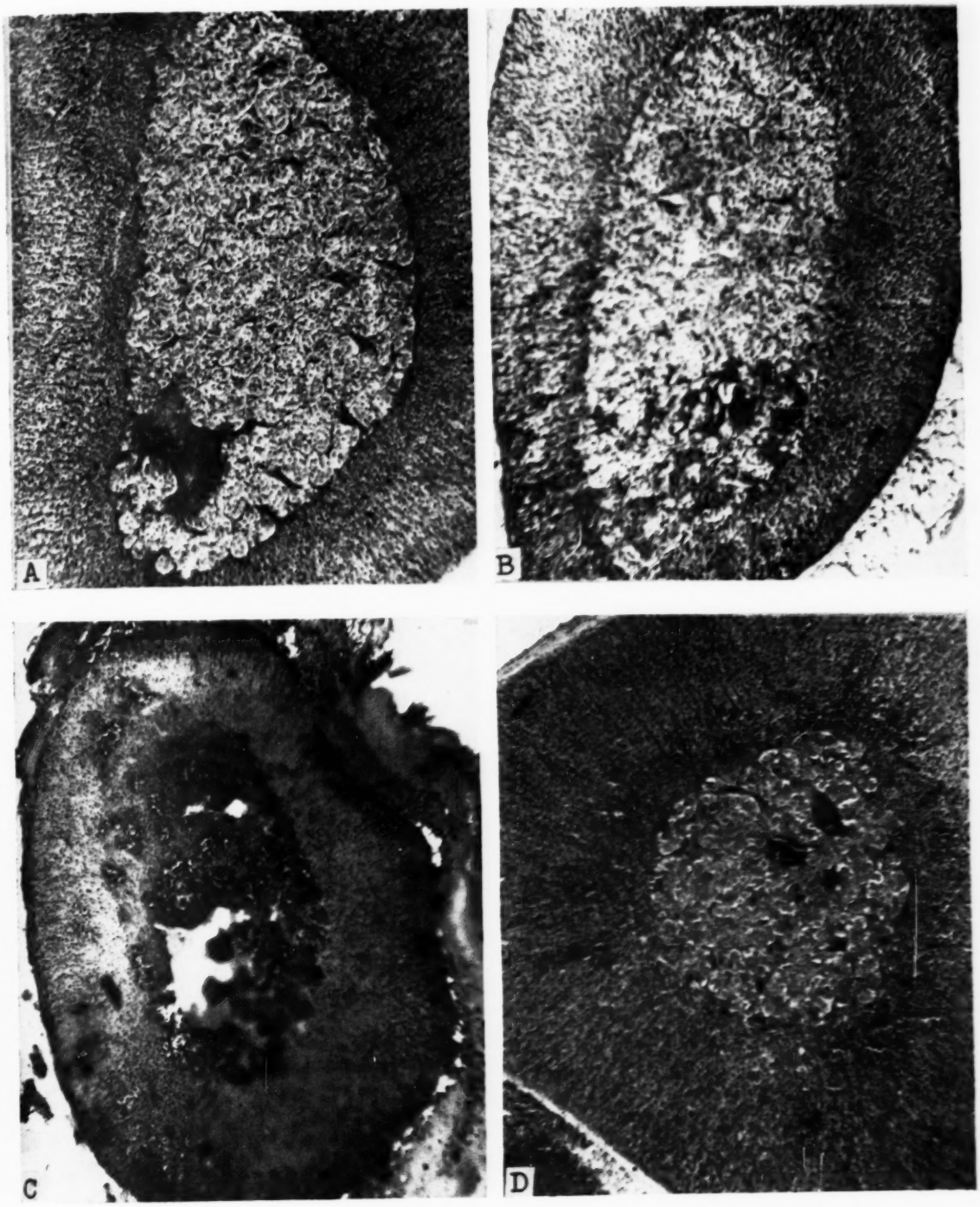

FIG. 5

DESCRIPTION OF FIGURE 6

FIG. 6.—Adrenal glands from female mice of C3H strain bearing spontaneous mammary tumors. A and B—cortex and medulla from untreated tumor bearing mouse; C and D —cortex and medulla from tumor bearing mouse injected with *S. marcescens* polysaccharide. E and F—cortex and medulla from tumor bearing C3H mouse injected with adrenal cortical extract only. Mag. × 440.

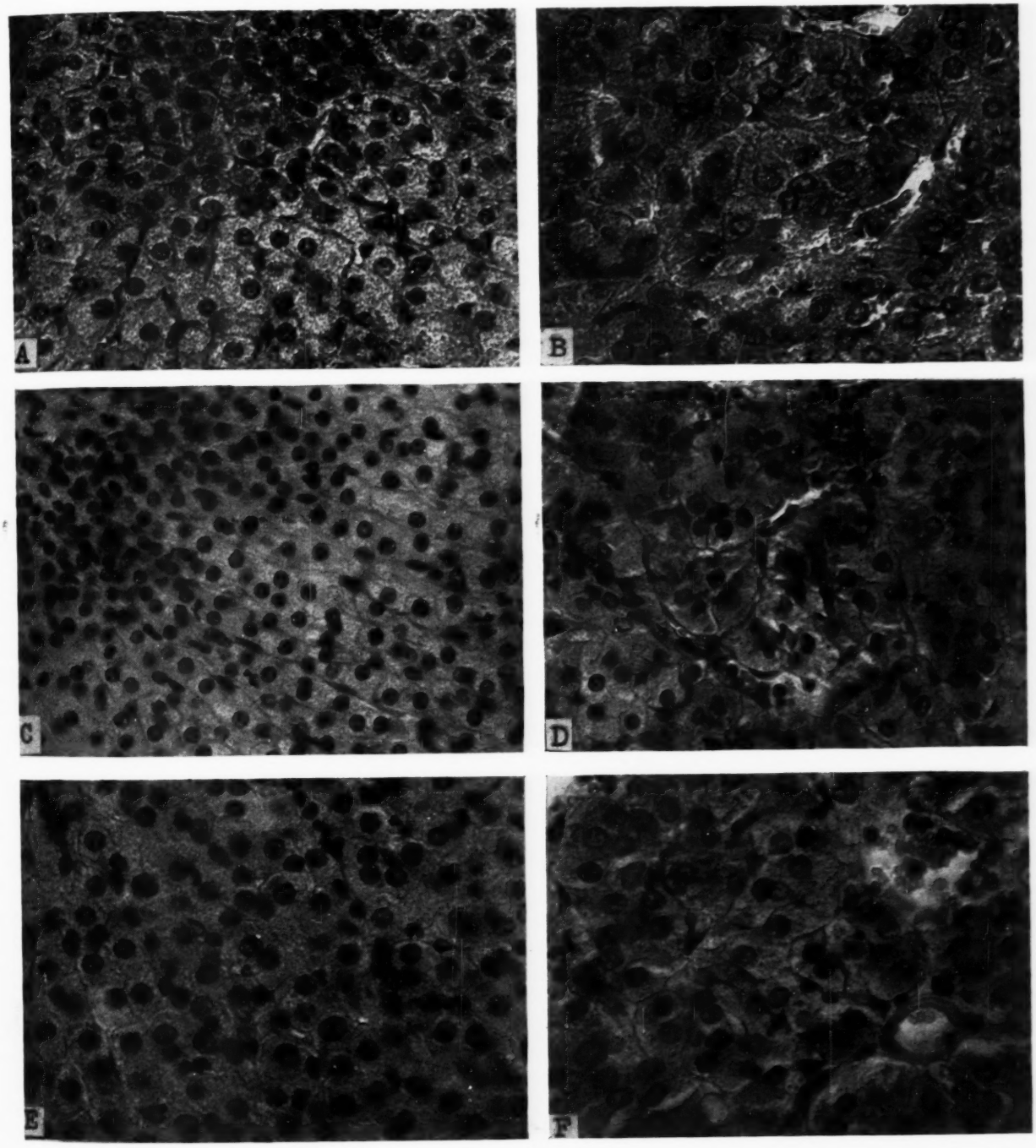

FIG. 6

Nucleic Acid Content in Intestines of Rats after Neutron Radiation

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It was reported in a previous paper (2) that whole-body x-radiation of rats resulted in decreased amounts of desoxyribonucleic acid, ribonucleic acid, structural proteins, and non-volatile ash in the cells of the crypts of Lieberkühn in the intestines. Results of a similar study of whole-body neutron radiation of rats are reported in the present paper.

MATERIALS AND METHODS

Male albino rats, approximately 200 gm. in weight, maintained on Fox Blox food (Allied Mills Co.) with water *ad libitum* were used. The rats were divided into 7 groups of 20 each; 10 of each group were irradiated and 10 were used as controls. Neutron irradiation was performed as described by Enns (3). Groups 1 to 5 were given 56 n and tissues were removed for examination at the following intervals after completion of irradiation: Group 1 immediately; Group 2, 4 hours; Group 3, 12 hours; Group 4, 24 hours; Group 5, 48 hours. Group 6 was given 100 n and tissues taken 24 hours later; Group 7 was given 200 n and tissues taken 24 hours later.

The rats in each group were paired according to weight: one of each pair was irradiated, the other served as control. The tissue to be examined was taken from the duodenum one-half inch below the pylorus. Tissue was removed from the irradiated and control rats of each pair at the same time and was carried through the fixation and dehydration process together. The fixative was absolute ethyl alcohol 3 parts and glacial acetic acid 1 part. Specimens from control and irradiated animals were embedded in close proximity in the same block of embedding medium so that they were cut at the same time. Comparison was always made between control and irradiated sections that were cut with the same stroke of the knife. Sections for studies by ultraviolet light absorption were cut 3 microns in thickness and were mounted on quartz slides without the use of adhesive. After deparaffination in xylol, the sections were placed in 95 per cent ethyl alcohol, then mounted with glycerol. Sections for microincineration were cut 7 microns in thickness and were mounted on glass slides without the use of adhesive. Sections for Feulgen staining were cut 10 microns in thickness and those for methyl green-pyronin staining 6 microns and were mounted on glass slides with albumin as adhesive.

A quartz optical system microscope in combination with a mercury-vapor lamp, monochromator, and 35 mm. camera were used to measure the ultraviolet light absorption by sections of tissues. The mercury light band 2654 A was used. Panatomic-X film was used and developed in Eastman Kodak D–19 developer. A 10 × ocular and a 2.5 mm. glycerine immersion objective were used. The magnification on the film was 195 ×. A minimum of 5 microscopic fields of each irradiated and control section were photomicrographed on the same strip of film using the same exposure and light intensity for each exposure. One frame on each film was exposed through a clear space between sections as a "background" for densitometric measurements. Densities of the photomicrographic images on the film were measured by use of a microdensitometer. Thirty to fifty readings were made on each of 5 fields and each "background." These data were used for calculation of extinction coefficients.

Feulgen stained sections were photomicrographed on microfile film and developed with Eastman Kodak developer D–9. Tungsten light and a green filter were used. Densitometric measurements on a scale comparable to that employed with the ultraviolet light absorption method were made and the data used for calculation of extinction coefficients.

Methyl green-pyronin stained sections were examined microscopically for differences in intensity of pyronin staining as a measure of the comparative amounts of cytoplasmic ribonucleic acid. Brachet (1) showed that pyronin is highly specific as a stain for ribonucleic acid.

Non-volatile inorganic ash was determined by densitometric measurements of intensities of dark-field photomicrographic images of microincinerated sections. The sections were incinerated at 600 to 625 ° C.

Photomicrographs of control and irradiated specimens to be compared were made with identical exposure and development times; thus differences in tone represent differences in absorption; the darker the tone, the greater the absorption.

RESULTS

The absorption of ultraviolet light (2654 A) was found to be reduced in the epithelial cells of the crypts of Lieberkühn 4, 12, 24, and 48 hours after the application of 56 n whole-body irradiation (Table I). In Group 1, taken immediately after completion of irradiation, the decreased absorption shown in the table is not considered significant; 5 specimens showed decreased absorption and 5 showed greater absorption than their controls. In Group 2, taken 4 hours after irradiation, 9 of 10 specimens from irradiated rats showed decreased absorption; in other groups specimens from all irradiated rats showed decreased absorption. Absorption was decreased in both the cytoplasm and nuclei, thus indicating decreased amounts of cytoploplasmic ribonucleic acid and nuclear desoxyribonucleic acid. The comparatively small amount of absorption by protein at this wave length and the minor effect of light dispersion by cell structures, both of which affect the photographic film and the densitometric measurements, were disregarded.

TABLE I: DECREASE OF ULTRAVIOLET LIGHT ABSORPTION BY CRYPTS OF LIEBERKÜHN IN THE DUODENUM OF NEUTRON-IRRADIATED RATS

Group No.	Neutron dose, n	Time interval, hours	Extinction coefficients average		Decreased absorption %
			Control	Irradiated	
1	56	0	0.914	0.867	5.1
2	56	4	1.059	0.887	16.2
3	56	12	1.000	0.817	18.3
4	56	24	0.857	0.690	19.5
5	56	48	0.921	0.768	16.6
6	100	24	1.043	0.792	24.1
7	200	24	1.075	0.706	34.3

There was a progressive decrease in absorption after 56 n up to 24 hours; the decrease was greater during the first 12 hours than during the second 12 hours. Neutron doses of 56, 100, and 200 n resulted in progressively greater decrease in absorption in 24 hours as the dose was increased.

Examination of tissue sections stained by the Feulgen technic (Table II), showed that there was a marked decrease in the amount of nuclear desoxyribonucleic acid (based on the assumption that the Feulgen reaction is specific for this acid) 24 hours after 56, 100, and 200 n and that the amount of change increased with the dose.

TABLE II: DECREASE IN AMOUNT OF DESOXYRIBONUCLEIC ACID IN CELL NUCLEI IN THE CRYPTS OF LIEBERKÜHN IN THE DUODENUM OF NEUTRON-IRRADIATED RATS. FEULGEN REACTION

Group No.	Neutron dose, n	Time interval, hours	Extinction coefficients, average		Decreased absorption %
			Control	Irradiated	
4	56	24	1.281	0.810	36.8
6	100	24	1.323	0.761	42.5
7	200	24	1.351	0.720	46.7

Microscopic examination of methyl green-pyronin stained sections showed decreased cytoplasmic pyronin staining in all groups except Group 1. The results agreed with those of ultraviolet light absorption methods.

Examination of the ash of microincinerated sections from Group 7 (24 hours after 200 n) showed a great decrease in the ash content of the crypts of Lieberkühn after irradiation. The average densitometer readings were: control 13.6; irradiated 35.4. Since the data are based on darkfield photomicrographs, the higher numerical value indicates the smaller amount of ash.

Morphological changes occurred in the crypts of Lieberkühn after irradiation. There was no change in cell size (based on area of nuclei in sections of tissue) or in the number of cells 4 hours and 12 hours after 56 n; however, 24 hours after 56 n, the number of cells had decreased by approximately 35 per cent and the average size of the nuclei had increased approximately 40 per cent. Twenty-four hours after 100 n, the number of cells had decreased approximately 44 per cent and nuclear size had increased by 40 per cent; 24 hours after 200 n, there was approximately a 50 per cent decrease in the number of cells and approximately a 45 per cent increase in nuclear size. Figs. 1 to 4 show these morphological changes and also the decreased absorption in the irradiated specimens.

DISCUSSION

Decrease in content of nucleic acids preceded the morphological changes and reduction in cell numbers, since at 4 and 12 hours after 56 n, nucleic acids were reduced in amount but cell number and nuclear size had not changed. However, 24 hours after irradiation, the morphological changes had occurred. Presumably, many cells in the crypts of Lieberkühn in the duodenum either were killed at time of irradiation or died later as the result of injury.

The effects observed are end results as a consequence of disruption of the established order. Determination of the primary effect of irradiation which initiates the sequence of destructive events will be difficult. Studies on depolymerization of nucleic acid by Taylor, Greenstein, and Hollaender (5) appear to be a step in the right direction.

The effects of neutrons were indistinguishable from those found after x-radiation (2). This was to be expected since electromagnetic radiation and neutrons do not themselves produce effects except by

virtue of the action of charged particles which they produce as secondary products (4). 56 n apparently produced less destruction, and 200 n more destruction in 24 hours than were observed 24 hours after 600 r. 100 n appeared to have approximately the same effect in 24 hours as did 600 r.

SUMMARY

Effects of whole-body neutron radiation of rats on the crypts of Lieberkühn have been studied by ultraviolet light absorption and other methods. It was found that neutron radiation caused a decreased ultraviolet light absorption in the crypts of Lieberkühn in the duodenum of rats; the decrease was found at various periods of time ranging from 4 to 48 hours after irradiation. Larger doses of neutrons produced greater effects. Involved in decreased absorption of ultraviolet light were ribonucleic acid and desoxyribonucleic acid. The amount of inorganic ash also was found to be reduced.

The effects of neutron radiation were similar to those previously found after x-radiation.

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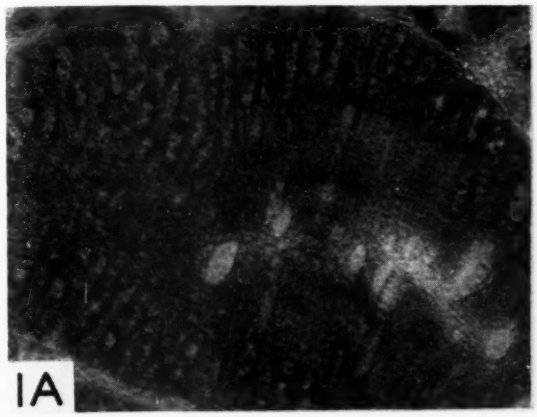
DESCRIPTION OF FIGURES 1 TO 4

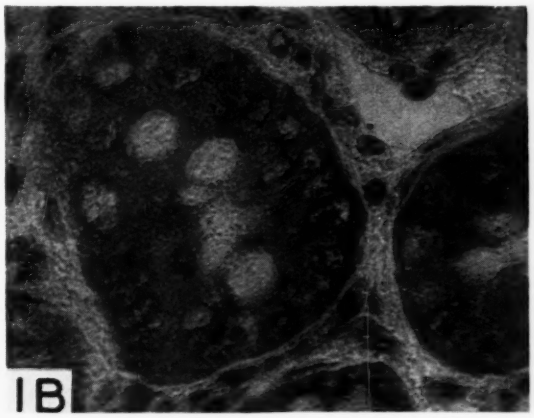
FIG. 1.—Ultraviolet light (2654 A) photomicrographs of crypts of Lieberkühn in sections from duodenum of (A) control and (B) neutron irradiated (56 n) rats 24 hours after irradiations. Mag. × 875.

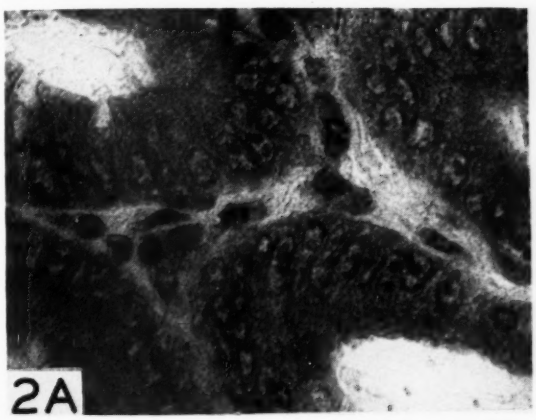
FIG. 2.—Same as Fig. 1, using 100 n.

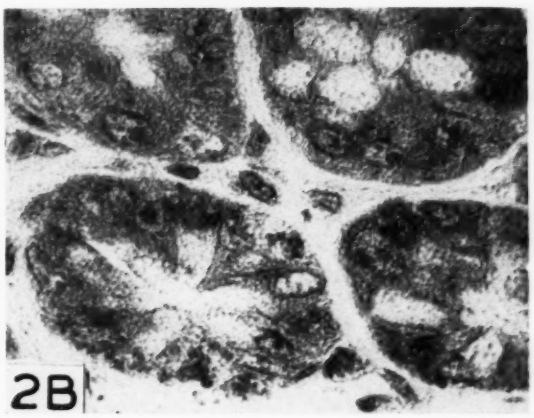
FIG. 3.—Same as Fig. 1, using 200 n.

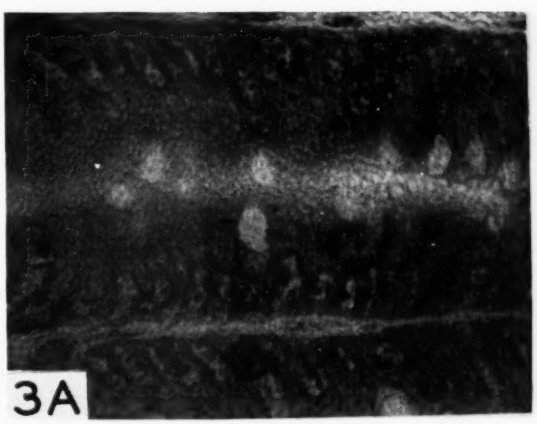
FIG. 4.—Photomicrographs of crypts of Lieberkühn in Feulgen stained sections taken from duodenum of (A) control and (B) neutron-irradiated (200 n) rats 24 hours after irradiation. Mag. × 1,130.

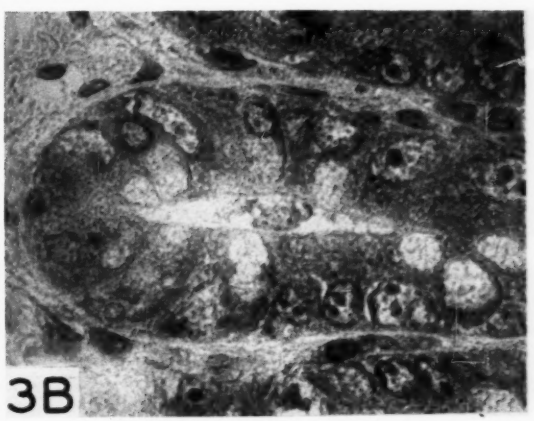


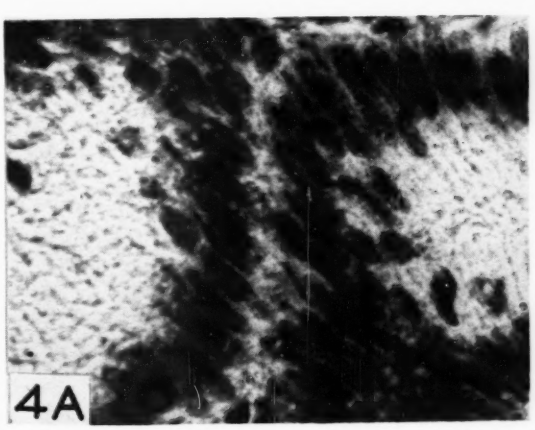


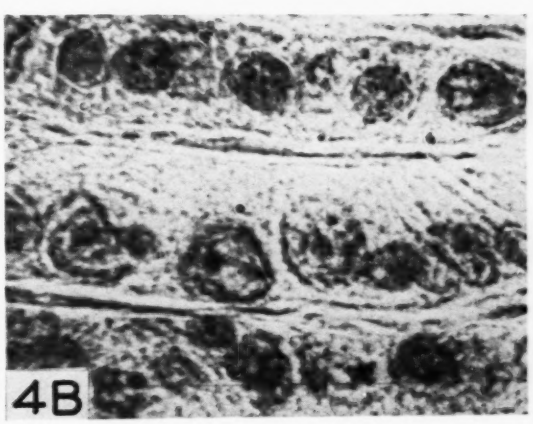












FIGS. 1–4

Induction of Malignant Tumors by Methylcholanthrene in Transplanted Uterine Cornua and Cervices of Mice

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Spontaneous malignant epithelial tumors of the uterine cervices and cornua rarely occur in mice of most strains. One stock of mice had a high incidence of malignant uterine or cervical tumors. Cancer of the uterine cornua has not been induced experimentally although malignant tumors of the cervix and vagina have been shown, by previous investigations, to occur in mice given estrogen for long periods of time, (1, 3, 4–6, 11); or to follow the applications of carcinogens or carcinogens combined with hormones (2, 13, 14). A systematic study of the action of methylcholanthrene on the genital tissue has not been done previously.

Since the later part of the 19th century many attempts to induce cancer by transplantation of adult and embryonic tissues have been reported. Nichol, 1904 (12) apparently was the first one to transplant various kinds of adult tissues, including normal and pregnant uterine epithelium. He noted that uterine epithelium was one of the adult tissues capable of proliferation after transplantation to another host; but no cancer developed in the transplants. Recent investigations by Greene (7), and Rous and Smith (15) have shown that carcinomas and sarcomas could be produced in transplanted embryonic tissue treated with methylcholanthrene. More recently Horning (10) induced cancer by transplanting adult prostatic tissue treated with methylcholanthrene.

The present study concerns the malignant tumors induced by methylcholanthrene in transplanted young adult uterine cervical or cornual tissues in mice.

MATERIALS AND METHODS

In the first experiment 104 mice of both inbred strains and hybrid stocks were used. Most of the mice were of weaning age but some of them were 1 or 2 months older. The uterine cervices and horns were removed under aseptic conditions. In some of the animals in which the vaginas were open at the time of operation mercurichrome was used for disinfection of the cervical tissue. Small crystals of methylcholanthrene were inserted into the excised crevices and horns and the tissues were then transplanted into other hosts, usually their brothers or sisters. The transplants were placed subcutaneously in the upper abdominal areas of the mice. Some of the animals received one graft; others two or more grafts. All of the mice were kept in an air-conditioned house and were fed a diet of Purina Fox Chow and water *ad libitum*. All the animals were examined twice weekly. The date of the first appearance of the tumor, as a visible or palpable mass of approximately 5 mm. in diameter, was recorded. The tumors were measured at intervals during the life of the animals and the gross size was recorded at autopsy. Unusual occurrences such as bleeding and ulceration were noted in all instances. Most of the animals were killed when the tumors reached a considerable size or when the general condition of the animals had declined and death of the animal was imminent. A few of the animals died, induced tumors contributing largely to their death. At autopsy the tumor tissue, regional lymph nodes, lungs, liver, kidneys and other organs, were preserved in Bouin's and formalin fixatives, and paraffin sections were made which were stained with hematoxylin and triosin routinely. Masson's trichrome and Laidlaw's reticulum stains have been used for demonstrating reticulum in some of the tumors. The induced tumors were transplanted in a number of instances and the transplanted tumors were carried through two or three generations.

In the second experiment a group of 18 mice was used for the study of the lesions shortly after transplantation. The animals were killed at weekly intervals up to the tenth week. The grafts were removed with the over- and underlying tissues and fixed in Bouin's solution. Two out of every 10 sections were mounted and stained with hematoxylin and triosin.

In the third experiment 25 mice were used to study the distribution of the methylcholanthrene as

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revealed by fluorescence microscopy. Most of the animals were killed at intervals of 24, 36, 48, 72 hours, etc., up to one week; others were killed 6 to 7 weeks after transplantation. The tissue removed was fixed in formalin and frozen sections were cut and mounted with glycerol. The light source used was a mercury vapor lamp, using a Ratten gelatin filter 2A to remove the longer rays. The presence or absence and the distribution of the crystals and fluorescence were recorded. Photomicrographs were taken of the fluorescent crystals and tissues, using a Leica camera and plus X film. Hematoxylin and triosin, Giemsa, trypan blue, acid fuchsin and methyelene blue stains were used for orientation of the lesions in these preparations. In addition the Sudan IV stain was used for the demonstration of fat substance in some of the specimens.

Cervical or uterine tissues were not transplanted without the insertion of carcinogen, because previous investigation in this laboratory revealed that no cancer developed in such transplanted uterine tissue in mice of the A strain (5). Small pieces of spleen, liver, ovaries, bladder and rectum were transplanted after similar treatment with methylcholanthrene.

OBSERVATIONS

Of the 104 mice of both inbred strains and hybrid stocks in the first experiment, 55 animals developed 61 tumors. The tumors consisted of 32 carcinomas and 29 sarcomas (Table I). More tumors arose from the transplanted cervical tissue and more tumors developed in mice of the A strain. No sex difference has been observed. In the present study the age of the recipients did not influence the incidence of tumor growth. The carcinomas appeared earlier than the sarcomas. About 65 per cent of the induced tumors developed before the end of the eighth week (Table II). In most instances at 3 to 5 days after transplantation an initial increase in size of grafts was observed. Some grafts disappeared later, others remained unchanged for some time or increased steadily in size. The rate of growth of the epithelial tumors was more constant and uniform whereas that of the connective tissue tumors was abrupt and rapidly progressive. Most of the tumors reached a size of 2 to 3 cm. in diameter within a period of 2 to 3 months. Invasion to the underlying muscle tissue was observed frequently. No metastases to the distant organs were observed, but lymphatic extension was noted in 2 animals. In the carcinomatous group the skin was invaded early, and this was followed by ulceration. Cystic change and hemorrhage have been observed in the methylcholanthrene-treated transplanted uterine horns.

The carcinomas were spheroid and nodular, usually invading the overlying skin and underlying muscle (Fig. 1). The cut surfaces were pale grey and slightly granular. Necrosis was noted in most tumors and grey-yellow atheromatous patches were seen in some tumors. The external surface of the sarcomas was smooth and rounded (Fig. 2). The tumor tissue was usually pink and fleshy although grey-red necrotic centers were often seen in the sarcomas.

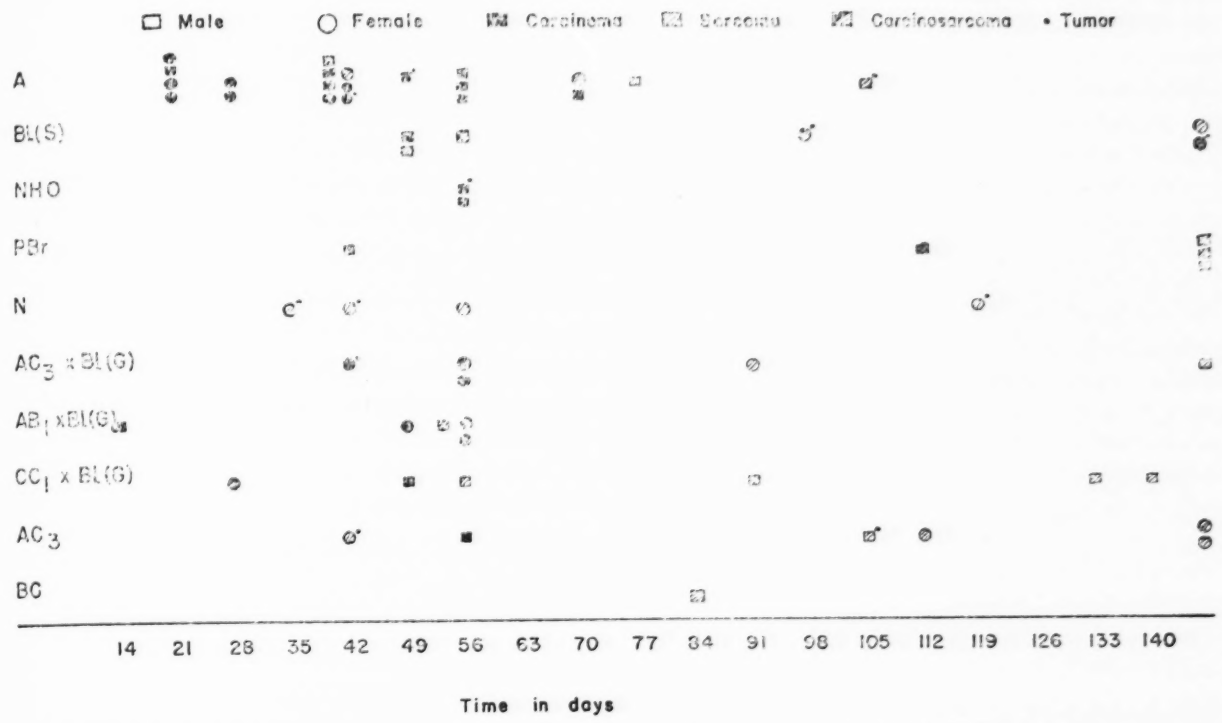
Histologically most carcinomas were of the epidermoid type, composed of irregular masses or sheets of more or less differentiated cells with an inconspicuous stroma. In many of the tumors the cancerous cells formed epithelial pearls or small cystic areas with mucoid material in the center (Fig. 3). The tumor cells in the peripheral portion were usually less differentiated and mitoses were more frequent. The tumor cells invaded the muscle fibers (Fig. 4). One adenocarcinoma and several adenoacanthomas arose in the transplanted uterine horns treated with methylcholanthrene (Fig. 5). The sar-

TABLE I: TUMORS IN METHYLCHOLANTHRENE-TREATED TRANSPLANTED UTERINE CERVICAL AND CORNUAL TISSUES OF MICE OF SEVERAL STRAINS AND HYBRID GROUPS

Strains and stocks of mice	No.	Sex		Average age at time of transplantation, days	Tissues transplanted		No. of mice with tumors	No. of tumors	Origin of tumors		Nature of tumors	
		M	F		Cervix	Horn			Cervix	Horn	Ca.	Sar.
A	20	10	10	27 (7) 62 (6) 217 (7)	18	23	7 (27) 16 4 (62) 5 (217)	21	18	3	16	5
BL	13	5	8	33	6	7	5	6	4	2	3	3
NHO	13	13	0	40	6	7	2	2	1	1	2	0
PBr	15	9	6	30	10	6	5	5	5	0	2	3
N	4	0	4	23	1	3	4	4	1	3	0	4
AC_2	11	5	6	32	6	5	6	6	4	2	1	5
$AC_2 \times BL(G)$	7	1	6	27	6	1	5	5	4	1	3	2
$AB_1 \times BL(G)$	6	2	4	32	6	0	5	5	5	0	3	2
$CC_1 \times BL(G)$	9	4	5	35	9	0	6	6	6	0	2	4
BD	6	6	0	150	1	5	1	1	1	0	0	1
Total	104	55	49		69	57	55 (30M) (25F)	61	49	12	32	29

TABLE II

The Latent Period of the Induced Tumors in Mice of Different Strains and Stocks



comas were for the most part composed of spindle cells; in some instances they were round, and in others, were pleomorphic (Fig. 6). In some tumors the epithelial elements were immediately surrounded by the neoplastic connective tissue, thus forming a picture of carcinosarcoma (Fig. 7). Other tumors consisted of a mixture of adenocarcinosarcomatous elements. Remnants of the cervices and horns were recognizable in the centers of some of the large tumors (Fig. 8). A few sarcomas developed in the control group and one of the transplanted bladders showed cancerous lesions.

Eight carcinomas and 4 sarcomas that arose in the transplanted uterine tissue were transplanted into other hosts. The transplants of the carcinomas were histologically identical with the original tumors. In some of the transplanted pleomorphic cell sarcomas the subsequent transplants were composed of spindle cells resembling fibroblasts.

A study of the serial sections from the grafted tissue removed shortly after transplantation, revealed that from the end of the second week on there was suggestive evidence of neoplastic growth of the epithelial cells of the cervical mucosa. In a hybrid mouse that was killed at the end of the second week, localized areas of small and irregular strands of epithelial cells had penetrated the basement membrane of the cervical mucosa and grown into the underlying tissue (Fig. 9). The irregularly stratified epithelium was partially cornified and surrounding areas filled with leukocytes and sloughed cells. These neoplastic changes were associated with inflammatory reactions in the stroma. The remaining portion of the cervical mucosa revealed either diffuse increase in thickness of the epithelial cells or no alteration. Sometimes necrosis of the epithelium was associated with cellular exudate. In a hybrid mouse that was killed at the fourth week the grafted cervical tissue exhibited definite localized areas of neoplastic change; in some areas the epithelial cells had lost their polarity, were deeply stained and had penetrated the basement membrane (Fig. 10). In association with these neoplastic cells small collections of polymorphonuclear cells were found among the superficial layers of the degenerated epithelial cells. Another animal of the A strain had 2 tumors 6 weeks after transplantation; one consisted of an epidermoid carcinoma arising from the grafted cervical tissues and measured 2 cm. in size. The other was composed of several small carcinomatous nodules. One of the 2 tumors was composed of epidermoid cells, apparently arising from the proximal portion of the uterine horn; the other growth showed initial neoplasia of the endometrium of the distal end of the horn. These tumors were composed of small and irregular cystic structures, lined by 2 or

more layers of cuboidal or cornified cells. The architecture of the stroma of the uterine horn tissue was recognizable. The lumen of the uterine horn toward the distal end was partly lined by a stratified epithelium.

At one week after transplantation fluid and cellular exudate had accumulated in the lumen of the grafted cervices, and localized areas of thickening of the epithelium had occurred. Groups of leukocytes in the lumen were arranged radially around structures resembling crystals. In the thickened epithelium disintegrated phagocytes and polymorphonuclear leukocytes were surrounded by both degenerating and proliferating epithelial cells.

The subcutaneous tissue of the host showed marked inflammatory reactions during the first or second weeks and then the inflammation gradually subsided. The epidermis of most of the hosts revealed no change, but in some instances the areas adjacent to the tumor were inseparable from the neoplastic tissue. In some animals the tumor tissue merged with the sebaceous glands. The adjacent mammary tissue of the female hosts usually remained unaltered but were hyperplastic in one animal.

The sections examined with the use of ultraviolet light showed both green-yellow fluorescence of the methylcholanthrene crystals and the blue fluorescence of the dissolved carcinogen in the lumen of the grafted tissue. In some instances the crystals were attached to the epithelium. In association with the rounded blue masses of fluorescence noted in the lumen were small light blue granules. In 5 animals small fluorescent granules were also present in the epithelium (Figs. 11 and 12). In one animal, killed at the end of one week, the transplant had attached firmly to the skin and fluorescence was observed in the sebaceous glands of the host's skin. Blue fluorescence was observed in the surrounding adipose tissue. Crystals and fluorescence decreased gradually toward the end of the first week; however, in two animals that were killed at the end of the sixth and seventh weeks respectively, a few crystals and small areas of fluorescence were noted in the grafted tissue. One of these 2 grafts showed a small cancerous lesion of the epidermoid type. The blood vessels and lymphatics and their contents in the transplanted tissues revealed no fluorescence. The lymph nodes, livers and gall bladder were examined in some instances and revealed no detectable fluorescence.

Acid fuchsin stain did not interfere with the visualization of the fluorescence of the methylcholanthrene; while preparations stained with Giemsa, methylene blue, or hematoxylin revealed decreased intensity or failed to show any fluorescence. Crystals could be seen in frozen preparations thus stained and mounted in glycerol without passing through the fat solvents. Some of the preparations were stained with hematoxylin and triosin after they had been studied under ultraviolet light in an unstained state (Fig. 11). The close relationship of the crystals and fluorescent substance, accumulated leukocytes and the local tissue could be determined. Using Sudan IV many fat droplets could be seen in the superficial layers of the epithelium and also in the cellular exudate.

DISCUSSION

The morphological evidence presented here indicates that malignant tumors, including carcinomas, adenocarcinomas, adenoacanthomas, carcinosarcomas and sarcomas arose in transplanted uterine cervical and cornual tissue treated with methylcholanthrene. Evidence was sufficient to establish the origin of the tumor tissue both in the grafted tissue removed shortly after transplantation and in the fully developed tumors. The histological characteristics of the epidermoid carcinomas were similar to the cancers seen in the untreated and estrogen-treated mice (3, 4, 6). Tumors of similar types appear to develop under the influence of different inciting agents.

Although approximately equal numbers of transplants of the uterine cervices and horns have been made, only a small percentage of the uterine cornual transplants showed malignant tumors. The reasons for the relative infrequency of the epithelial tumors of the endometrium are unknown but the epithelium of the endometrium may be refractory to the carcinogen used or the quantity of methylcholanthrene may have caused excessive necrosis of the epithelium.

The tendency for the initial neoplastic lesions to be localized in certain areas of the epithelium in the treated transplant tissues has been observed. This was also true in the gastric cancers (18).

Due to the frequent association of these initial lesions with the accumulated leukocytes it has been assumed that there might have been some relationship between the applied carcinogen, cellular exudate and the reaction of the local tissue. Fluorescence of carcinogens in skin has been studied (16, 17). Graffi (8, 9), using carcinogenic hydrocarbons dissolved in glycerin and serum, demonstrated fluorescence in many cultured tumors and normal tissues including leukocytes. As a result of the present fluorescence study it seems that the localization of the initial neoplasia was the result of a concentration of crystals of methylcholanthrene in the trans-

planted tissue. It is also possible that the cellular elements in the exudate might have facilitated or altered the action of the carcinogen. The presence of fluorescence in the fat tissue has been observed by many investigators. The significance of this observation is not certain.

The high incidence of these induced carcinomas in mice of the A strain is of interest. There is no evidence that mice of the A strain are more susceptible to spontaneous cancers of the genital tissue. The increased susceptibility to tumor formation may be partly due to the fact that the mice of the A strain are highly inbred animals in which the homologous grafts showed a higher degree of survival. It might also be possible that these mice react to the carcinogen in a different manner than the other strains do. Further investigation is necessary to elucidate this problem.

SUMMARY AND CONCLUSION

Fifty-five of the 104 mice of both inbred strains and hybrid stocks developed 61 malignant tumors in transplanted uterine cervical and horn tissues treated with methylcholanthrene. The malignant tumors consisted of epidermoid carcinomas, adenocarcinomas, adenoacanthomas, carcinosarcomas and sarcomas. Fewer tumors arose from the transplanted cornual tissue than from cervices. More cancers developed in mice of the A strain than the other strains used.

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DESCRIPTION OF FIGURES 1 TO 6

FIG. 1.—Gross appearance of an induced carcinoma that developed from the transplanted cervical tissue in a mouse of the NHO strain.

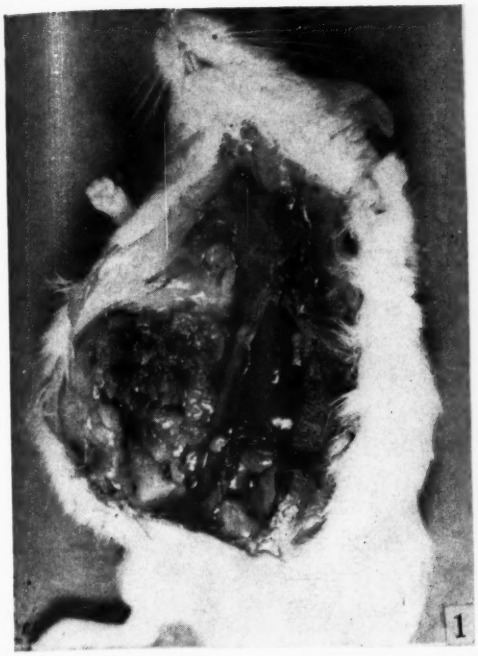
FIG. 2.—An induced sarcoma with rounded and smooth surface is seen in the lower chest wall of a hybrid mouse.

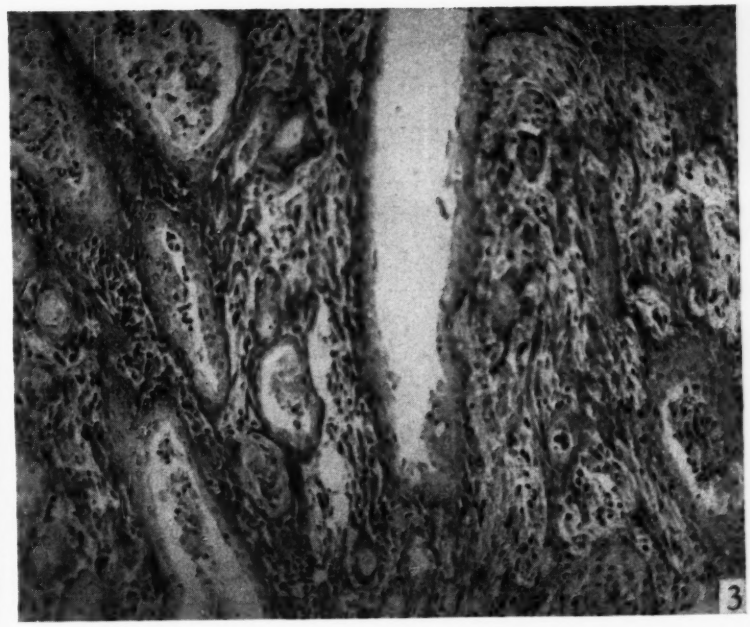
FIG. 3.—Photomicrograph of an epidermoid carcinoma arising from the transplanted cervical tissue. The cervical mucosa was seen in the center of the tumor.

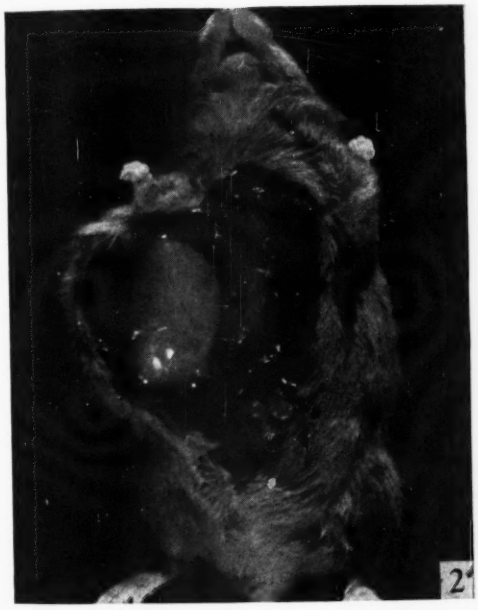
FIG. 4.—Photomicrograph of an epidermoid carcinoma arising from the transplanted cervical tissue of a mouse of the A strain. Small and irregular solid strands of tumor cells invaded the muscle fibers.

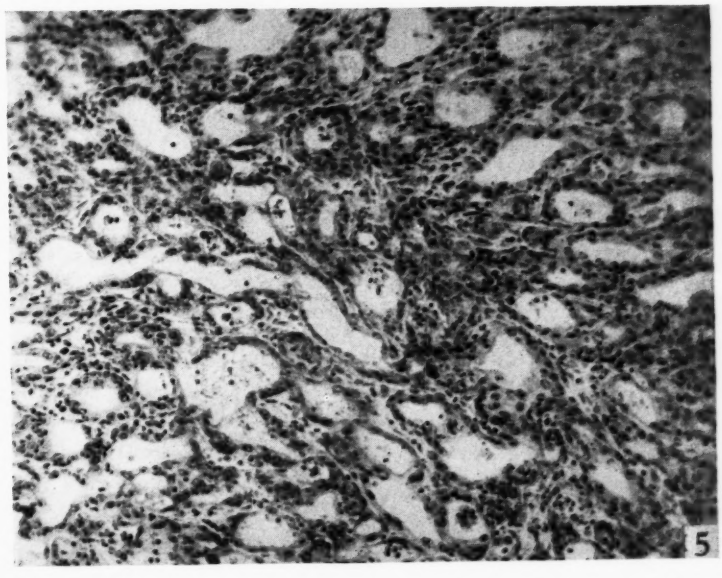
FIG. 5.—Photomicrograph of an adenocarcinoma of a hybrid mouse. The tumor is composed of irregular glandular structures. Many mitoses are seen.

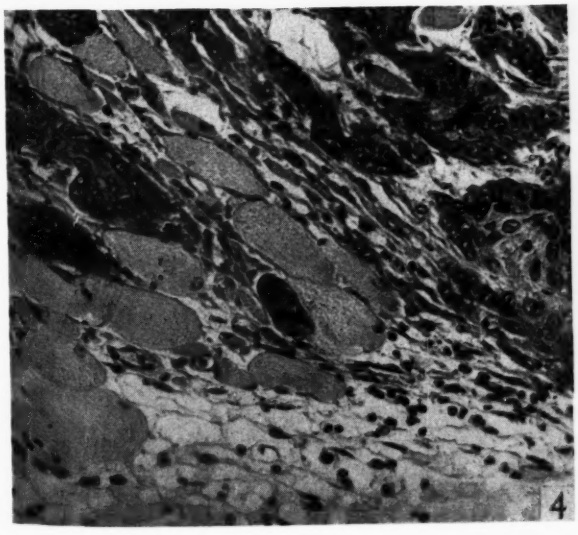
FIG. 6.—Photomicrograph of a pleomorphic cell sarcoma.

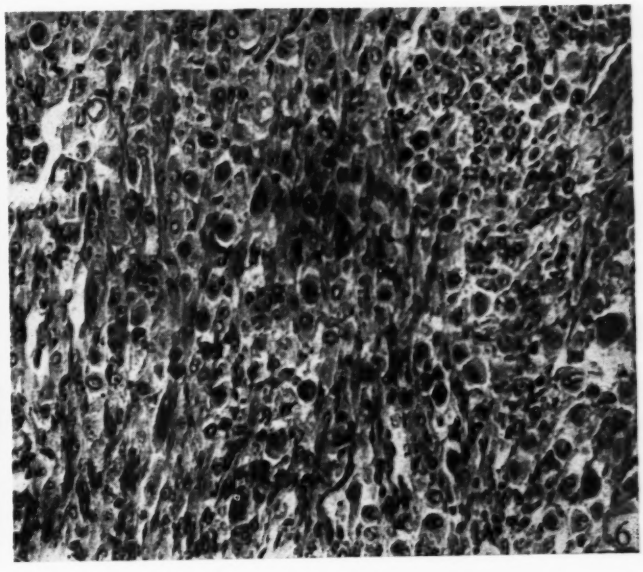












FIGS. 1–6

DESCRIPTION OF FIGURES 7 TO 12

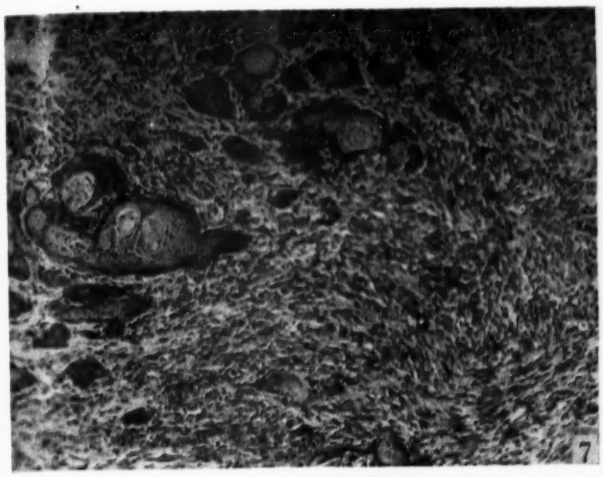
FIG. 7.—Photomicrograph of a carcinosarcoma arising from the transplanted cervical tissue in a hybrid mouse.

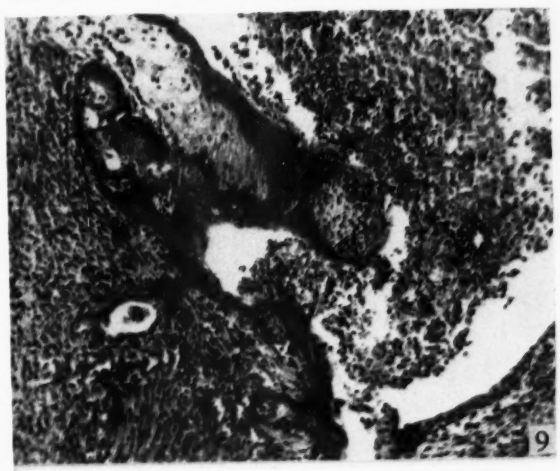
FIG. 8.—Low power view of the same tumor revealing the original architecture of the transplanted cervical tissue.

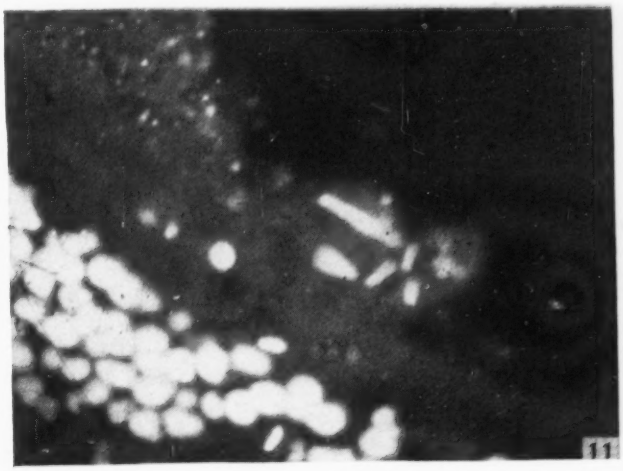
FIGS. 9 and 10.—The localized initial neoplasia of the epithelium in the transplanted cervical tissue.

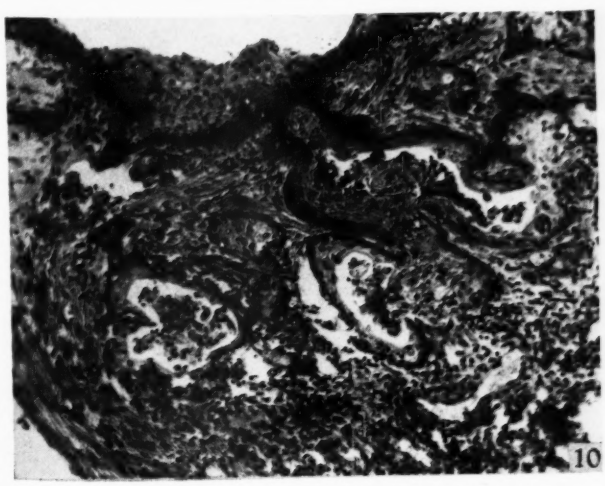
FIG. 11.—Photomicrograph of a preparation examined with the ultraviolet light. The spindle shaped crystals were green yellow in color and the small granules in the inflamed epithelium were blue and fluorescent.

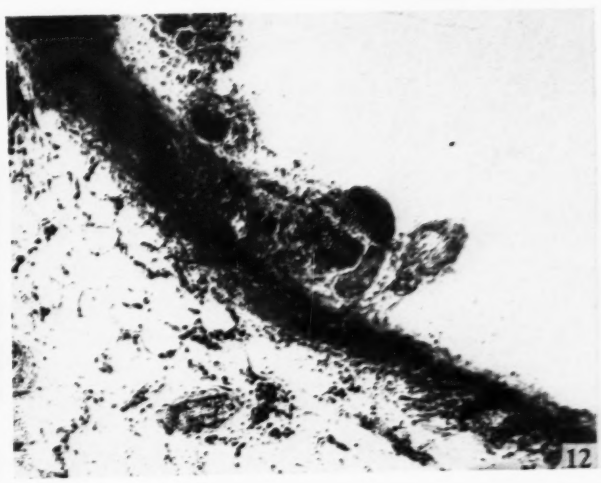
FIG. 12.—The same preparation stained with hematoxylin and triosin. Accumulation of polymorphonuclear leukocytes is seen in the areas where the crystals and fluorescent granules were present.











FIGS. 7–12

National Research Council Appoints Subcommittee on Oncology

The Committee on Pathology of the National Research Council has appointed a Subcommittee on Oncology.

The members are:

Dr. Shields Warren, *Chairman*
Dr. Balduin Lucké
Dr. Fred Stewart
Dr. Harold Stewart
Dr. Arthur P. Stout
Dr. Milton C. Winternitz
Dr. Howard T. Karsner, *Chairman of Committee on Pathology, ex officio*

Brig. Gen. Raymond C. Dart, Director of the Army Institute of Pathology, is cooperating with the Committee and making the Institute's facilities and resources available and is providing office space for the permanent secretary.

The objectives of the Subcommittee are:

1. Improvement in the teaching of oncology;
2. Dissemination of information on oncology to clinical pathologists, students, and teachers of oncology;
3. The establishment of criteria for diagnosis of tumors;
4. The simplification of terminology by recommending a single term for each tumor and listing separately the appropriate synonyms.

The Subcommittee expects to work with existing agencies to promote clarity and unity in tumor nomenclature and classification.

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